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ACUTE INHALATION OF EXPLOSIVELY DISSEMINATED CARBON FIBERS IN RATS

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13. ABSTRACT *(Maximum 200 words)*

Carbon fibers are lightweight, high tensile strength synthetic strands used commercially and in military applications for aircraft and electromagnetic obscuration. Fibers with respirable diameters <3.5 μm and lengths >10 μm will deposit by interception in the bronchi. Recent XM81 grenade field studies indicated that explosive dissemination produced fibers with diameters <3.0 μm and lengths <100 μm, making some of the fibrous smoke respirable. Carbon fibers were explosively generated to determine if the resultant aerosol is respirable and if it adversely affects rodents. Groups of 344 male Fischer rats were exposed to one of the three concentrations of carbon fibers for 30 min. Air-exposed and fuse/fuel-exposed rats served as the controls. Exposed rats and respective control groups were submitted for bronchoalveolar lavage; biochemical, physiological, and pathological evaluation at 24 hr and 14 and 90 days post exposure. Also, SEM analysis was used to characterize the size range of the fibers and to determine fiber deposition in trachea and lung tissues. Aerosol samples were collected for mutagenicity testing. Initial results indicated the presence of fiber fragments and combustion products in the respirable range. However, there were no adverse changes in the biological responses of the rats from short-term exposure to XM81 grenade aerosols.

14. SUBJECT TERMS

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PREFACE

The work described in this report was authorized under Project No. 1C463627DE79, Smoke and Obscurants. The work was started in November 1991 and completed in April 1992. The experimental data are contained in laboratory notebooks 85-0162, 88-0117, 90-0094, 91-0044, 91-0101.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institute of Health Publication No. 85-23, 1985, as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council (Washington, DC). These investigations were also performed in accordance with the requirements of AR 79-18, "Laboratory Animals, Procurement, Transportation, Use, Care, and Public Affairs," and the Laboratory Animal Use and Review Committee (LAURC), U.S. Army Chemical Research, Development and Engineering Center (CRDEC),* which oversees the use of laboratory animals by reviewing for approval all CRDEC research protocols requiring laboratory animals. This project, assigned LAURC Protocol No. 22091000A269, was approved by the U.S. Army Chemical Research, Development and Engineering Center Laboratory Animal Use Review Committee. Every reasonable attempt was made to control bias throughout the experiment. All chamber analysis data, toxic observations, and animal weights were recorded in official CRDEC notebooks. Physiology and lavage data were generated on hard copy outputs from automated instruments. This data was analyzed and entered in official CRDEC notebooks. All other associated raw data (statistical printouts, necropsy incidence tables, etc.) was stored in CRDEC, Research Directorate, Toxicity Division archives.

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Acknowledgments

The authors acknowledge Bernardita Infiesto for her assistance in the analyses of the lavage fluid; Dr. Erica Petersen, Physics Division (CRDEC), for her scanning electron microscopy (SEM) analysis of the generated materials; Dr. John Petrali, U.S. Army Medical Research Institute of Chemical Defense, for SEM tissue examination; and Steve Anthony, Toxicity Division (CRDEC), for the mass spectroscopy analysis; Fred Lee for monitoring the mutagenicity contracts.

QUALITY ASSURANCE

This study, conducted as described by Protocol 22091000A269, was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792 (effective 17 Aug 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase inspected	Date	Date reported
Dosing via inhalation*	18 Dec 1990	20 Dec 1990
Lavage, chemistry, necropsy*	3 Jan 1991	4 Jan 1991
Draft Pathology Report	16 Mar 1992	23 Mar 1992
Data & Final Report	20 Jan 1993	21 Jan 1993

* These phases were inspected during the companion study, as described by Protocol 22091000A265, which was conducted just prior to this effort.

To the best of my knowledge, the methods described were the methods followed during the study. The report was determined to be an accurate reflection of the raw data obtained.

Dennis W. Johnson 21 Jan 93

DENNIS W. JOHNSON
QA Coordinator, Research & Technology

STUDY INSPECTION FORM (GLP)
(40 CFR 792)

Title (Nature) of Study : Contract report (draft) from Pathology Associates Inc.

Protocol #: 22091000A269

Test Substance: Carbon Fibers Explosively Detonated.

Sponsor: Drew Farenwald

Study Director: Dr. Sandra A. Thomson

Date Study Began: 5 August 1991

Phase Inspected: Review of draft contract report for GLP compliance.

Date of Inspection: 16 March, 1992

Findings:

This draft report has been reviewed by the Toxicology Quality Assurance Unit for Good Laboratory Practice (GLP) compliance as required by 40 CFR Part 792 Toxic Substance Control Act, Good Laboratory Practice Standards, 1989. The findings from the review are that the report is an accurate representation of the data presented, and as far as can be determined the contractor conformed with GLP regulations.

Kenneth P Cameron 23 MAR 92

Kenneth P. Cameron
GLP Inspector

cf: Dr. Harry Salem
Dr. Sandra A. Thomson

CONTENTS

LIST OF FIGURES AND TABLES

Figure

Table

ACUTE INHALATION OF EXPLOSIVELY DISSEMINATED CARBON FIBERS IN RATS

1. INTRODUCTION

Carbon fibers are lightweight, high tensile strength synthetic materials used in the fabrication of graphite laminate composites. Commercial uses for these composites include sporting equipment, prosthetic devices for humans, and structural components of aircraft.[1,2] Also, their smooth outer construction has led to increased use in military aircraft. The electromagnetic obscuration capability of carbon fibers offers another military application, which is one of the obscurant materials in the bispectral XM81 smoke grenade. Carbon fibers can be manufactured from petroleum pitch or polyacrylonitrile (PAN).[3] PAN is the commonly used precursor yielding fibers with a higher purity and was the material used in this study.

Carbon fibers can be disseminated by mechanical or explosive methods. Fiber diameter has been identified as the determining factor for respirability, based on modeling experiments with equivalent aerodynamic diameters.[4] The upper limit of respirability, for fiber diameter has been established at 3.5 μm.[5,6] A previous inhalation study, using mechanical dissemination of 3.5 μm diameter carbon fibers (aspect ratio approximately 1000), demonstrated that fibers of this dimension were not respirable in rodents.[7] Carbon fibers used in industry and in the XM81 grenade have a diameter of 7-8 μm, which is considered outside the respirable range. However, the aerosol cloud analyses from recent field trials of the XM81 grenade, using the NIOSH 7400 method, indicate that explosive dissemination produced fibers with diameters <3.0 μm and lengths <100 μm.* This would make some of the fibers inhalable and possibly respirable. Additionally, this mode of dissemination presents a potential for synergistic effects with the combustion by-products.

Although there have been numerous studies addressing the health hazards from carbon fiber exposure, few inhalation studies have been conducted,[8] and none of the studies adequately described the particle size distribution. In addition, several *in vitro* and *in vivo* studies comparing PAN and pitch based carbon fibers[9] have shown positive mutagenicity tests with the pitch based fibers, but similar tests were negative for the PAN-based fibers.[8,10]

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*CPT Joseph Terra, U.S. Army Biomedical Research and Development Laboratory, May 1993, unpublished data.

The purpose of this study, therefore, was to determine if respirable fiber fragments are produced from the explosive dissemination of carbon fibers and if there are any adverse biological responses in rodents from inhalation of the resultant aerosol. Particle size distribution and morphology, along with mutagenicity assays, were other important objectives included in this study.

2. MATERIALS AND METHODS

2.1 Materials.

The PAN-based, 7.5-μm diameter carbon fibers (Hercules Incorporated, Wilmington, DE, Magnamite Graphite Fiber AU4, CAS #7782-42-5) containing 92-99.7% carbon were fabricated into discs (2.3" diameter X 0.25" thickness, Figure 1). Sixteen discs or approximately 300 g are positioned in each grenade.

The XM81 grenades were obtained from the U.S. Army Chemical Research, Development and Engineering Center (CRDEC),* Munitions Directorate and were composed of the following items:

- Expulsion Charge (Propellant - Class 6 Black Powder)
- Delay Detonator
- Transfer Lead
- Booster Lead
- Burster
- Carbon Fiber Discs

A detailed description of the grenade components is in Appendix A.

2.2 Animal Utilization/Husbandry/Necropsy.

Male Fischer 344 rats were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival, the rats were housed in individual, suspended stainless steel cages in the Toxicology Division animal care facility. Housing conditions were maintained at a temperature of 20 to 24 °C, 30-70% relative humidity, and a 12 hr light/dark cycle. Water and certified rodent chow were provided *ad libitum*. Rodent management, handling, and utilization was in accordance with NIH

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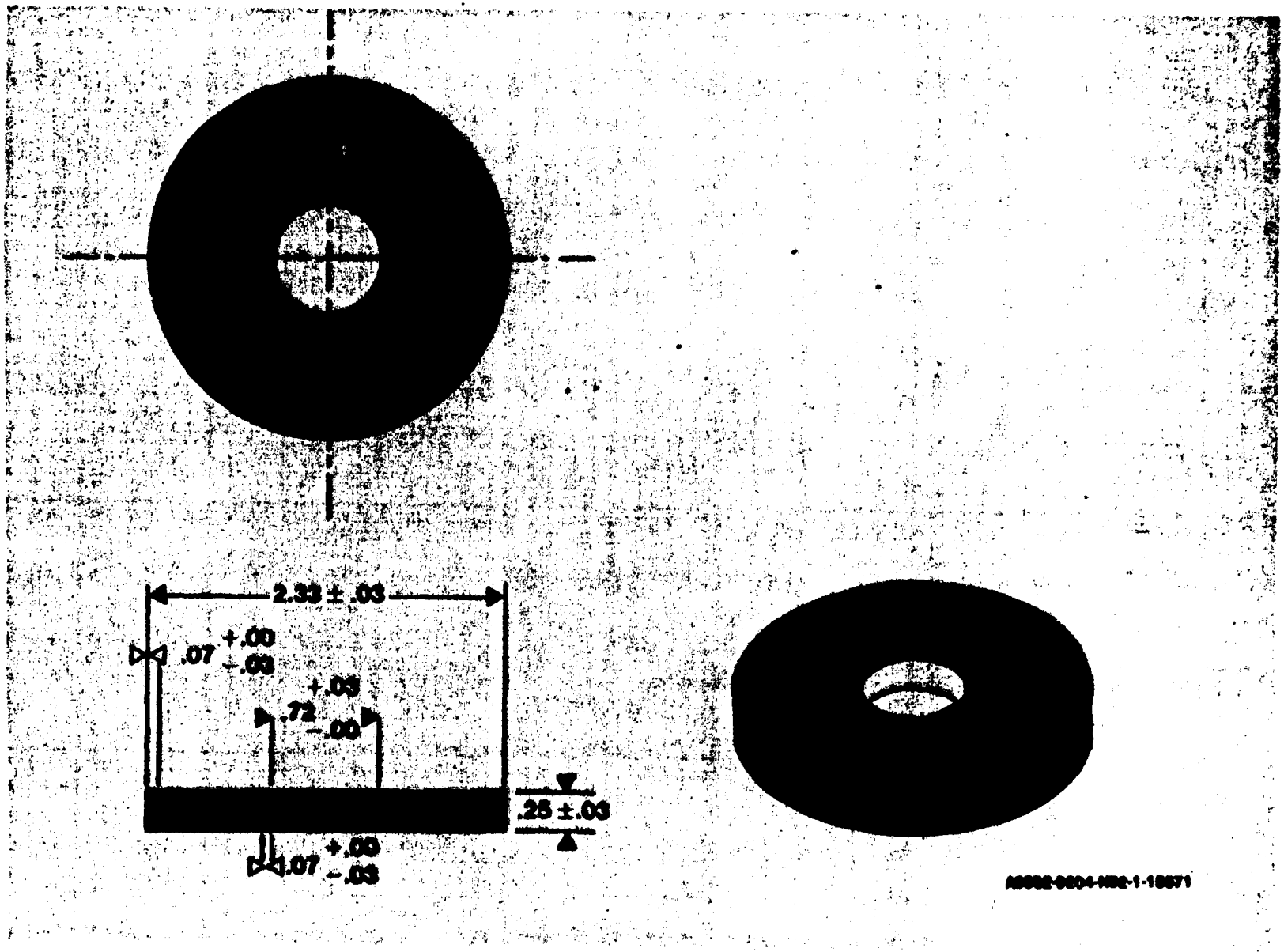


Figure 1. Carbon Fiber Disc Size

publication 85-23, "Guide for Care and Use of Laboratory Animals."[11] During the prescribed 7-day quarantine period, animals were examined by the Chief of the Veterinary Services Branch and determined to be in good health (Appendix B). Daily observations and weekly weighings were made throughout the study.

One week before the scheduled start date, the rats were weighed, tattooed, and randomly placed into groups. On the day of exposure, a climate controlled vehicle was used to transport the rats to the exposure chamber. The rats were returned in the same vehicle to the animal facility immediately after exposure.

Groups of 12 or 36 male rats were exposed by whole body inhalation to one of three concentrations of carbon fiber aerosol for 30 min. Because previous studies with pyrotechnic/explosive devices have shown particulate and vapor phase contribution from the burster components,[12,13] another group of rats was exposed to fuse/fuel only. All exposed rats and a respective group of air-exposed controls were evaluated for physiological, broncho-alveolar lavage (BAL), and histopathological changes at 24 hr, 14 days, and 3 month post exposure (PE). Animals were also submitted for scanning electron microscope (SEM) evaluation to determine deposition sites of the carbon fragments. Two hundred four rats were used. Listed below is the utilization breakdown.

Number of Rats

	CONTROL	FUSE/ FUEL	LOW CONC.	MID CONC.	HIGH CONC.
0 hr PE					
SEM	1	2	0	0	3
24 hr PE					
Pathology	6	6	6	6	6
Phys/BAL	6	6	6	6	6
SEM	1	2	0	0	3
14 days PE	6	6	6	6	6
Pathology	6	6	6	6	6
Phys/BAL	1	2	0	0	3
Sem					
3 month PE	6	6	6	6	6
Pathology	6	6	6	6	6
Phys/BAL	1	2	0	0	3
SEM					

Because pathological evaluation required the animals be terminated, separate animal groups were needed for pathology, physiology/bronchoalveolar lavage, and SEM evaluation. However, the BAL and physiology measurements were conducted on the same animals.

2.3 Chamber Operation and Sample Collection.

The rats were exposed whole-body in a 300 L Hinners type chamber. A 4-in. diameter PVC pipe was extended from the exposure chamber to the center of a 20 m^3 chamber. Full-size grenades were detonated in the 20 m^3 chamber, and the resulting aerosol cloud was drawn into the 300 L exposure chamber. Incorporated into the PVC pipe, between the two chambers, was a "Y" fitting that allowed dilution air to be introduced into the aerosol stream. Exposure concentrations were achieved by using a combination of flow restriction and/or dilution. The high concentration was attained by drawing undiluted atmosphere from the large chamber through a 1 ½" orifice. The same restriction along with dilution air was used for the mid concentration. The low concentration was attained by using a ¾" orifice along with dilution air. Air control and fuse/fuel exposures were conducted under the same flow conditions as the high concentration.

Aerosol concentration was measured gravimetrically by drawing chamber air through glass fiber filter pads. After the animal exposure began, samples were taken at 5, 15, and 25 min. Also, a continuous sample was collected during the entire animal exposure to determine a time-weighted average concentration (TWA).

Combustion gases from the exposure chamber were monitored using Matheson-Kitagawa (Matheson Gas Products, Secaucus, NJ) precision gas detector tubes. Samples were drawn at the same intervals as the concentration samples using a 2-L syringe. From this sample, specified volumes were collected through the detector tubes using a 100 mL Matheson-Kitagawa sampling pump. After waiting the specified gas reaction time, colorimetric indicators inside the tubes indicated the concentration of the specific gas. Gases analyzed during calibration and found to be below detectable limits were HCN, HF, methyl chloroform, NO_2, NO_X and SO_2. Gases analyzed routinely during all exposures were carbon monoxide (CO), carbon dioxide (CO_2), formaldehyde (HCHO), and ammonia (NH_3).

2.4 Particle Size Analysis.

2.4.1 Cascade Impactor.

Particle size samples were taken with a 10-stage cascade impactor (Anderson Samplers, Model 2210, Atlanta, GA). The cascade impactor was operated at 0.25 CFM for the mid and

high concentrations, and at 0.35 CFM for the fuse/fuel exposure and low concentration. Sample time varied with concentration. At these flow rates, particle size could be measured between 18 and 0.16 μm (mid and high concentration) and 15 and 0.25 μm (fuse/fuel and low concentration). Material was collected on lightly greased stainless steel substrates beneath each stage. Particle size sample data were analyzed by log-normal regression, least squares method, of particle size versus cumulative relative mass. The mass median aerodynamic diameter and geometric standard deviation (σ_g) of the aerosol was determined during the calibration and exposure portions of the study.

2.4.2 SEM.

To document the morphological changes of the airborne particles, conventional SEM was conducted on selected test specimens, as well as the graphite materials before dissemination. Samples were collected from the exposure chamber on 25 mm polycarbonate membrane filters with 0.1 μm pores (Nucleopore Corporation, Pleasanton, CA). For each test grenade, samples were taken at the same intervals as the concentration samples. Samples from the control exposure and room air were also collected for 17 samples. Each sample was collected at a flow rate of 5 L/min for 5 s.

Specimens for particle sizing were prepared by cutting wedges from the filters, attaching them to aluminum (Al) SEM sample mounts with double sided tape, painting the edges with colloidal carbon paste, and sputtering with gold. Fiber specimens before dissemination were prepared by removing a cluster from a disk and attaching these to an Al specimen mount with colloidal carbon paint. Fine particles associated with the packed fibers were isolated by gentle shaking over an Al mount. Sputter coating was not required for this sample.

Particle sizing was accomplished using a JEOL 35CFM SEM (JEOL, Peabody, MA) equipped with a lanthanum hexaboride filament, operating at 15 KeV (Kilo electron Volts) accelerating voltage, and a particle recognition and counting program written by Tracor Northern Instruments (Noran Instruments, Middleton, WI). The program identifies every feature producing secondary electron intensity above a given threshold as a particle and uses control of the electron beam, based on feedback from the electron detector, to measure 8 chord diameters. The program reports diameters, area coverage, and a shape factor for each particle. These data are then used to produce particle size distribution histograms based on particle numbers.

2.5 Physiological Measurements.

At 24 hr, 14 days, and 3 month PE, rats were anesthetized with sodium pentobarbital (40 mg/kg) by intraperitoneal

injection. Two respiratory parameters, flow and transpulmonary pressure, were determined directly from each animal.

Respiratory flow was measured through a tracheal catheter connected to a Fleisch pneumotachometer. A Validyne differential pressure transducer (Validyne, Engineering Corporation, Northridge, CA) attached to the pneumotachometer by tubing converts the flow to the proper signals for the Buxco Pulmonary Mechanics Analyzer (Buxco Electronics, Incorporated, Troy, NY).

For transpulmonary pressure, a catheter was inserted into the esophagus, approximately to the level of the thoracic inlet. The catheter was connected to one arm of a Statham differential pressure transducer (Statham Laboratories, Incorporated, Hato Rey, PR). Transpulmonary pressure (taken as the difference between esophageal pressure and airway pressure derived from a lateral tap at the distal end of the endotracheal tube) is used for all calculations.

The flow and pressure signals were processed in a Buxco Pulmonary Mechanics Analyzer. These parameters were recorded from the analyzer: flow, transpulmonary pressure, tidal volume, compliance, resistance, respiratory rate, and minute volume.

Compliance is a standard physiological method of assessing the overall lung and thoracic elasticity. Compliance is measured as the ratio of the tidal volume to the pressure change between end expiration and end inspiration. Restrictive pulmonary diseases (e.g., fibrosis, silicosis) result in decreases in compliance due to a stiffening effect that increases the work of breathing. Resistance is a measure of the pressure difference required per unit of flow change. Inhalation of dusts/fibers may lead to an increase in airway resistance. Compliance and resistance were measured as indicators of functional impairment.

2.6 BAL.

Immediately following the pulmonary measurements, the esophageal catheter was withdrawn and the lavage procedure started. The lung washing technique consists of introducing a volume of saline (0.015 mL/g body weight) into the lung and immediately withdrawing the saline until a slight pressure is noticed on the syringe plunger. Two lung washings are done in this manner in rapid succession. The lung washings are combined and centrifuged at 300 g for 10 min at 4 °C.

Following centrifugation, the supernatant fluid was separated from the pellet. The pellet was resuspended in 1 mL 50% bovine serum albumin, and total cell counts were taken on a Coulter Counter Model ZBI (Hialeah, FL). A differential cell

count was made using a modified Wright-Giemsa Stain. The cell pellet was resuspended in Hank's buffered saline. The macrophage concentration was determined in a hemocytometer and cell viability determined via the trypan blue exclusion test.[14]

The supernatant lavage fluid was assayed for total protein with the BioRad® protein assay, and for enzymatic activity of lactate dehydrogenase (LDH), alkaline phosphatase (ALKP), and ß-glucuronidase (ß-Glu). The LDH and ALKP were determined on an Abbott VP Series II (Irving, TX), using an Abbott analysis kit. The ß-Glu was assayed using a Sigma Chemical Company kit (St. Louis, MO).

2.7 Histopathology.

At the appropriate intervals, rats scheduled for pathology were euthanized with carbon dioxide and necropsied. Total body weight and selected organ weights (adrenals, brain, kidneys, liver, lungs, testes) were recorded as part of the necropsy and their tissue prepared for light microscopic examination by Pathology Associates, Incorporated (Frederick, MD) in accordance with Contract No. DAAA15-92-D-0009. Pathology Associates, Incorporated, evaluated the tissues for histopathologic deviations.

2.8 SEM Tissue Examination.

At 0 hr, 24 hr, 14 days, and 3 month PE, animals were delivered to Dr. John P. Petrali of the U.S. Army Medical Research Institute of Chemical Defense (MRICD) for SEM tissue evaluation. Animals were anesthetized upon arrival with 1 mL sodium pentobarbital (50 mg/mL) intraperitoneally. The thoracic cavity was opened by midline excision and the entire respiratory system removed with the heart affixed. The trachea was intubated through the rima glottis of the larynx with a fixing catheter attached to a perfusion apparatus for drip infusion of fixative. With the lung unit floating in a bath of fixative, 50 mL of 1.6% formaldehyde and 2.5% glutaraldehyde in 0.1 sodium cacodylate (pH 7.34, 190 mOsm) was dripped from a height of 30 cm. Total fixation time was 24 hr at room temperature.

Following fixation, the unit was removed from the perfusion apparatus and the following dissections performed. The trachea was longitudinally bisected along the cartilaginous rings to display the mucosal surface for analysis. The distal portion of the trachea was dissected to present the superior surface of the carina. Lung parenchyma was dissected along the right and left major/primary bronchi to include surrounding alveoli.

All selected tissues were processed for SEM. Specimens were dehydrated in graded ethanol, critical-point dried in

liquid CO_2 at 42 °C and at 1300 psi and sputter coated with gold/palladium to a thickness of 16 nm. The specimens were then mounted on aluminum specimen holders with aluminum paste and viewed in an AMRAY 1820 SEM.

Additionally, some high concentration samples were examined by x-ray microanalysis to identify particle composition. Specimens and selected time frames were analyzed with a Delta Class III KEVEX Fisons Microanalyzer (Fisons Instruments, San Carlos, CA). X-ray spectra were acquired for 100 s for each particulate. The peaks were automatically identified, escape peaks removed, and background subtracted. This analysis was qualitative only.

2.9 Mutagenicity Assays.

Extracted samples of the exploded carbon fibers were submitted to Integrated Laboratory Systems (Research Triangle Park, NC) under Contract No. DAAA15-91-0093 for testing in the *Salmonella typhimurium*/microsome reverse mutation assay (Ames test), rodent bone marrow micronucleus assay, and the chromosome aberrations assay in Chinese hamster ovary (CHO) cells.

2.10 Data Analysis Plan.

Respiratory physiology and BAL data were analyzed according to the statistical "decision tree" described by Gad and Well.[15] Bartlett's test for homogenicity of variance was used, which was followed by the analysis of variances (ANOVA). Non-parametric data was analyzed by the Kruskal-Wallis nonparametric ANOVA.

3. RESULTS

3.1 Test Atmosphere Characterization.

3.1.1 Exposure Concentrations.

The airflow rate through the 300 L chamber was maintained at 463 ± 22 L/min. The aerosol concentrations of each exposure are listed in Table 1, and the results of the combustion gas monitoring are listed in Table 2.

3.1.2 Particle Sizing.

3.1.2.1 Cascade Impactor.

The MMAD and σ_g are in Table 3.

Table 1. Aerosol Concentrations of Explosively Disseminated Carbon Fibers from the XM81 Grenade (30-min exposure)

		Serial Filter Samples (mg/m^3)				TWA*
Date	Group	5 min	15 min	25 min	Average	mg/m^3
10/2/91	Fuse/Fuel	65	23	24	37	42
	Low Conc.	57	36	20	37	40
	Mid Conc.	223	82	49	118	119
	High Conc.	346	127	66	179	190
10/7/91	High Conc.	282	119	67	156	166

*Time weighted average

Table 2. Combustion Gas Results from XM81 Grenade Detonation

Group	CO (ppm)	CO_2 (%)	HCHO (ppm)	NH_3 (ppm)
STEL*	400	0.05	2	35
TLV - TWA**	50	0.15	1	25
Control	<5	0.1	<1	<5
Fuse/Fuel	33	0.1	7	6
Low Conc.	5	0.09	<1	<5
Mid Conc.	14	0.09	<1	12
High Conc.-1#	22	0.1	1.3	18
High Conc.-2##	38	0.06	<1	6

* American Conference of Governmental Industrial Hygenists (ACGIH) Short Term Exposure Level
** ACGIH Threshold Limit Value - Time Weighted Average
# First high concentration exposure
Second high concentration exposure

Table 3. Particle Size Results from Explosively Disseminated Carbon Fibers

Cascade Impactor Particle Size			
		MMAD**	σ_g♣
Date	Group		
10/2/91	Fuse/Fuel	0.99	6.58
	Low Conc.	3.36	3.40
	Mid Conc.	4.46	3.55
	High Conc.	3.68	3.08
10/7/91	High Conc.	3.69	3.29

** Mass Median Aerodynamic Diameter (micrometers)
♣ Geometric Standard Deviation

3.1.2.2 SEM.

Figures 2 and 3 are examples of the histograms provided by the particle counting and recognition program. All counts included a minimum of 300 particles and in most cases, more than 800 particles. Results of particle size distributions are displayed as percentage plots of the total particles in each size bin series. To increase the size bin resolution, it was necessary to count each area twice with each count addressing identical areas of the specimens. Data from the ranges 0.1 - 2.0 μm and 2.0 - 10.0 μm were joined and histograms calculated to cover the entire range from 0.1 - 10.0 μm. No particles larger than 10.0 μm were observed.

The particle size distribution is reported as the number of particles falling in a specific range of some equivalent size measurement. With the sizing method used, this equivalent is the average diameter as projected in two dimensions. Another correlation of interest is that which is between particle sizes and shape factors, or aspect ratios. The distributions of shape factors throughout the size bins were evaluated and no correlation could be made. The SEM particle analysis was provided by Dr. Erica Petersen, and the complete results have been submitted for publication as a separate CRDEC technical report.[16]

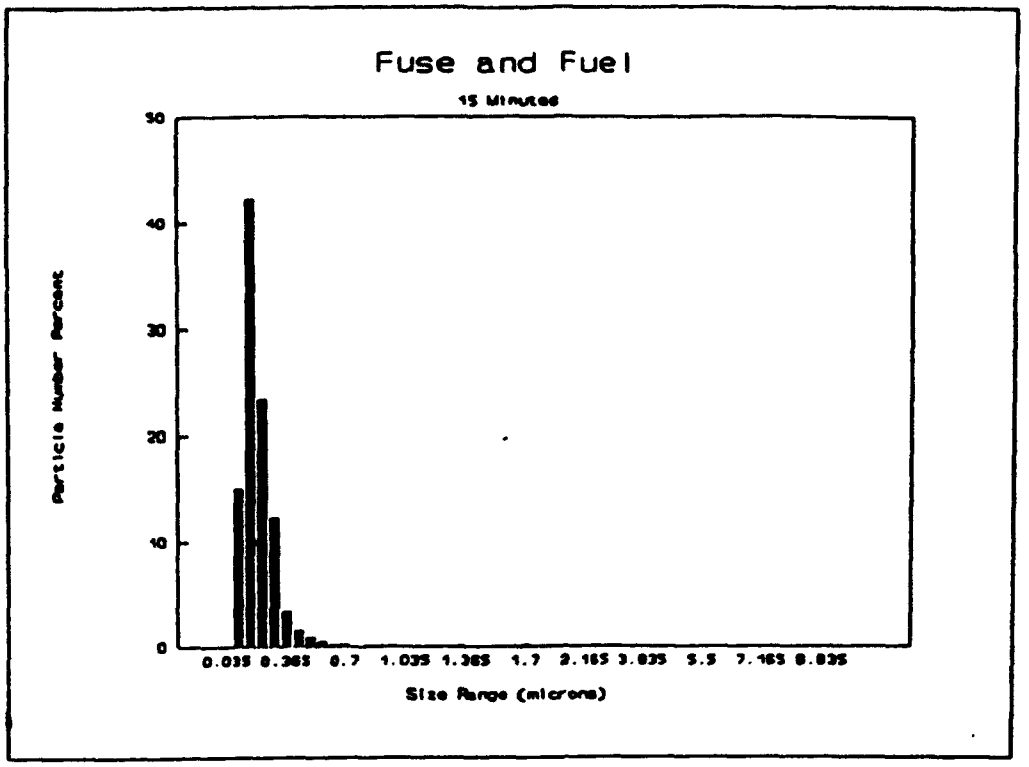


Figure 2. SEM Particle Size Distribution from XM81 Grenade Fuse/Fuel Detonation (t + 15 min)

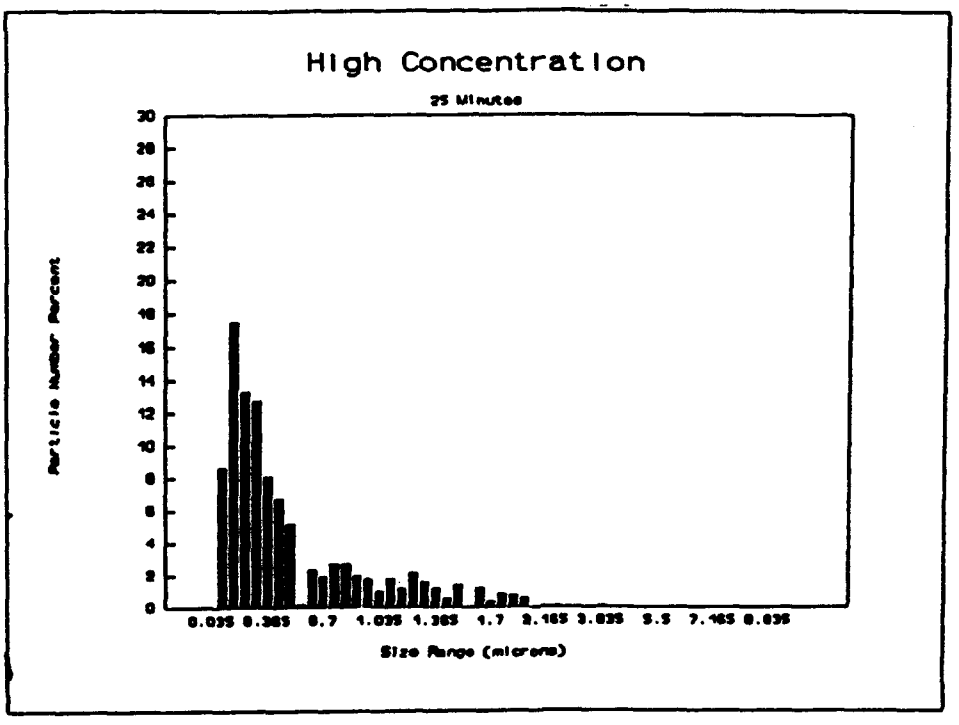


Figure 3. SEM Particle Size Distribution from XM81 Grenade Detonation (t + 25 min)

3.2 Toxic Sign Observations.

Rats exposed for 30 min to explosively disseminated carbon fibers did not exhibit any adverse toxic effects. The rats did not appear to be over-heated, stressed, or uncomfortable. Control and exposed rats displayed similar behavior upon removal from the holders. All exposed rats gained weight at the same rate as the control animals.

3.3 Physiological and BAL Responses.

The results of the physiological and BAL evaluations are presented in Tables 4, 5, and 6. There were no statistically significant differences in the physiology data between the exposed and control rats. The ALKP from the 24 hr PE high concentration animals was the only statistically significant BAL parameter.

3.4 SEM Tissue Evaluations.

Tissue samples from 24 animals were examined by SEM. No particulates were found in any of the control animals. Particulates were observed in the trachea and bronchus samples from the fuse/fuel and high concentration animals at 0 hr, 24 hr, and 14 days PE. At 3 month PE, particulates were located in the trachea of the high concentration animals. In addition to particulate imaging, a large proliferation of inflammatory cells, probably macrophages, were found on the mucosal surface of the trachea of all high concentration animals at 3 month PE.

The x-ray microanalysis of selected high concentration specimens revealed the presence of carbon and silicon particles. Carbon peaks were acquired at 0.282 KeV and silicon peaks at 1.740 KeV. Carbon particles were most prevalent in the zero hour PE animals, and silicon particles were most prevalent at 3 month PE. Appendix C contains the reports, photomicrographs, and spectra of the observed particles.

3.5 Histopathology.

There were no gross lesions observed in rats exposed to the carbon fibers or fuse/fuel. However, microscopic examination revealed the presence of brown/black pigment within alveolar macrophages of the rats exposed to both carbon fibers and fuse/fuel. By 3 months PE, the pigment was cleared from the fuse/fuel group, and there were fewer pigment laden macrophages in the carbon fiber exposed rats. No toxic or inflammatory reaction other than phagocytosis of carbon fiber pigment was present. At 3 months PE, small foci of granulomatous inflammation were present in some of the eyes of the rats exposed to carbon fibers. None of the material could actually be identified as fibers but

Table 4. Respiratory Physiology Results After Acute Inhalation of Carbon Fibers from the XM81 Grenade

Group	Weight (g)	Flow	Pleur. Press.	Tidal Volume	Comp-liance	Resis-tance	Resp. Rate	Minute Volume
24 Hr PE	(Average ± Standard Deviation, n = 6)							
Control	243	16.2	7.60	1.48	.228	.179	99.4	181
	12	8.8	.63	.57	.078	.095	6.4	62
Fuse/Fuel	232	16.8	8.82	1.41	.202	.216	94.6	166
	7	6.3	2.11	.39	.107	.175	11.2	53
Low Conc.	229	14.1	7.85	1.41	.221	.215	78.7	125
	6	2.5	1.64	.33	.051	.070	15.2	19
Mid Conc.	234	15.0	8.57	1.37	.209	.271	101.1	167
	7	7.5	2.44	.57	.134	.199	45.7	82
High	235	13.4	7.94	1.36	.213	.227	84.4	138
Conc.	6	1.8	1.68	.25	.084	.073	11.8	36
Bartlett	NS	SIG	NS	NS	NS	NS	SIG	NS
ANOVA	NS	NA	NS	NS	NS	NS	NA	NS
Kruskal-Wallis	-	NS	-	-	-	-	NS	-
14 Days PE	(Average ± Standard Deviation, n = 6)							
Control	265	20.3	8.21	1.71	.260	.164	112.6	235
	10	2.5	2.01	.34	.091	.103	23.9	45
Fuse/Fuel	253	19.1	8.58	1.60	.237	.226	129.6	204
	16	7.1	1.68	.37	.098	.129	72.4	113
Low Conc.	266	18.1	6.56	1.74	.551	.205	114.2	249
	6	5.9	.86	.34	.533	.084	16.1	61
Mid Conc.	273	21.5	7.65	1.69	.276	.167	112.1	237
	7	1.9	1.16	.25	.095	.058	20.5	72
High	256	19.9	7.38	1.36	.289	.151	116.2	233
Conc.	17	4.5	1.80	.37	.065	.053	26.9	41
Bartlett	NS	SIG	NS	NS	SIG	NS	SIG	NS
ANOVA	SIG	NA	NS	NS	NA	NS	NA	NS
Kruskal-Wallis	-	NS	-	-	NS	-	NS	-
Dunnett's	NS	-	-	-	-	-	-	-

Table 4. Respiratory Physiology Results After Acute Inhalation of Carbon Fibers from the XM81 Grenade* (Continued)

Group	Weight (g)	Flow	Pleur. Press.	Tidal Volume	Comp-liance	Resis-tance	Resp. Rate	Minute Volume
3 Month PE	(Average ± Standard Deviation, n = 6)							
Control	330	25.2	6.24	1.97	.415	.142	131.9	333
	18	3.0	.60	.21	.049	.016	11.9	35
Fuse/Fuel	349	30.1	6.74	2.21	.441	.148	114.9	319
	24	4.0	1.95	.49	.121	.064	27.5	68
Low Conc.	341	27.5	7.83	2.41	.429	.166	112.9	347
	15	4.0	1.74	.29	.123	.058	14.7	32
Mid Conc.	338	31.9	7.01	2.35	.460	.131	123.6	369
	14	6.9	1.22	.38	.169	.024	27.4	74
High	322	29.8	6.78	2.22	.495	.133	123.3	328
Conc.	14	5.1	2.39	.49	.127	.056	40.5	61
Bartlett	NS	NS	NS	NS	SIG	SIG	NS	NS
ANOVA	NS	NS	NS	NS	NA	NA	NS	NS
Kruskal-Wallis	-	-	-	-	NS	NS	-	-

*Flow is mL/s; press. is cm H_2O; Tidal vol. is mL; compliance is mL/cm H_2O; resistance is cm H_2O/mL/s; min vol. is mL/min.

Table 5. BAL* Fluid Analysis from Rats Exposed to Grenade Disseminated Carbon Fibers

	ß-Glu (Sigma U/mL)	LDH (IU/L)	Alk Phos (IU/L)	Protein (µg/mL)
24 Hr PE	(Mean ± Standard Deviation, n = 6)			
Control	3.62	32.5	71.4	305
	.30	17.0	6.2	44
Fuse/Fuel	4.42	30.7	65.8	352
	1.73	18.9	14.9	177
Low Conc.	3.52	34.7	66.1	344
	.95	18.9	16.2	37
Mid Conc.	3.90	39.8	56.3	351
	.92	18.9	3.4	45
High Conc.	3.86	33.4	54.6	281
	.82	19.3	6.6	29
Bartlett's	SIG	NS	SIG	SIG
ANOVA	-	NS	-	-
Kruskal-Wallis Nonparametric ANOVA	NS	-	SIG	NS
Distribution Free Multiple Comparison			Ctrl vs F/F - NS Ctrl vs LC - NS Ctrl vs MC - NS Ctrl vs HC - SIG	
14 days PE				
Control	4.32	21.7	65.2	340
	1.88	10.8	11.9	130
Fuse/Fuel	4.85	25.6	71.1	441
	1.12	9.1	7.7	198
Low Conc.	4.47	37.0	66.9	452
	2.01	25.7	19.6	226
Mid Conc.	4.17	20.7	62.2	334
	.73	3.2	7.4	52
High Conc.	4.37	28.4	61.1	367
	.83	11.1	9.2	63
Bartlett's Test	NS	SIG	NS	SIG
ANOVA	NS	-	NS	-
Kruskal-Wallis Nonparametric ANOVA	-	NS	-	NS

Table 5. BAL* Fluid Analysis from Rats Exposed to Grenade Disseminated Carbon Fibers (Continued)

	ß-Glu (Sigma U/mL)	LDH (IU/L)	Alk Phos (IU/L)	Protein (µg/mL)
3 Month PE	(Mean ± Standard Deviation, n = 6)			
Control	2.90	32.0	58.7	285
	.64	14.1	3.8	89
Fuse/Fuel	3.79	33.6	64.2	259
	.53	19.2	5.3	79
Low Conc.	3.23	30.2	55.1	239
	.91	17.2	8.9	51
Mid Conc.	3.20	30.4	50.8	297
	.60	11.4	11.9	78
High Conc.	3.26	33.2	51.4	346
	.85	7.8	17.8	52
Bartlett's Test	NS	NS	SIG	NS
ANOVA	NS	NS	-	NS
Kruskal-Wallis Nonparametric ANOVA	-	-	NS	-

*BAL - Bronchoalveolar Lavage

Table 6. Cytological Summary of BAL from Rats Exposed to Grenade Disseminated Carbon Fibers

	WBC* x10³	Total x10⁴	Macrophages %	Lymphocytes %	PMN** %
24 Hr PE	(Mean ± Standard Deviation, n = 6)				
Control	1.52	4.58	99	1	0
	.29	0.80	1	1	0
Fuse/Fuel	1.55	2.20	100	0	0
	.54	1.25	1	0	0
Low Conc.	1.63	4.11	99	1	0
	.39	.30	1	1	0
Mid Conc.	1.85	5.63	99	1	0
	1.36	2.24	1	1	0
High Conc.	2.18	4.42	99	1	0
	.90	.56	1	1	1
Bartlett's	NS	NS	-	-	-
ANOVA	NS	NS	-	-	-
14 Days PE					
Control	1.17	6.52	97	3	0
	.35	1.86	1	2	0
Fuse/Fuel	1.72	5.44	98	2	0
	.60	.82	2	2	0
Low Conc.	1.38	4.53	98	2	0
	.48	2.24	1	1	0
Mid Conc.	2.04	4.30	97	1	2
	.63	.94	2	1	1
High Conc.	1.67	4.31	98	2	0
	.56	1.15	1	1	0
Bartlett's	NS	NS	-	-	-
ANOVA	NS	NS	-	-	-
3 Month PE					
Control	1.97	5.59	95	2	3
	.78	2.78	8	2	1
Fuse/Fuel	2.18	6.15	97	3	0
	.74	1.41	2	1	0
Low Conc.	2.40	6.20	98	2	0
	.81	.97	2	1	0
Mid Conc.	2.27	5.11	97	2	1
	1.71	1.12	1	1	0
High Conc.	1.21	5.42	98	2	0
	.23	2.10	2	1	0
Bartlett's	NS	NS	-	-	-
ANOVA	NS	NS	-	-	-

*White blood cell
**Polymorphonuclear neutrophils

appeared as amorphous dust. Refer to Appendix D for the complete summary from Pathology Associates.

3.6 Mutagenicity Assays.

Extracts of the exploded carbon fibers were tested in the Ames, Mouse Micronucleus, and the CHO Chromosome Aberration Assays. The CHO results were positive in the presence of metabolic activation while the other results were all negative. The extracts of the explosively disseminated carbon fiber do not appear to be either mutagenic or clastogenic as tested in these studies. Refer to Appendix E for the complete summary.

4. DISCUSSION

Groups of male Fischer 344 rats were exposed to three concentrations of explosively disseminated carbon fibers for 30 min. Additional control groups of rats were exposed to air or the fuse/fuel components. At 24-hr, 14-day, and 3-month PE, the rats were evaluated for pulmonary mechanics, BAL, and histopathological changes. There were no compound-related mortalities and no significant changes in pulmonary mechanics, BAL, or histopathology. None of the rats from the high concentration groups exhibited any pulmonary lung lesions or significant morphologic changes. This demonstrated that the high level short term exposures did not overwhelm the lung clearance mechanisms.

Ammonia (NH_3), CO_2, CO, and HCHO were measured during each of the exposures. The HCHO concentration during the fuse/fuel exposure (7 ppm) was the only gas to exceed the ACGIH short-term exposure Level (STEL). The HCHO TLV-TWA is 1 ppm, and the STEL is 2 ppm. These HCHO levels may not be relevant, because these tests were conducted in a closed chamber, while actual field disseminations may have shown a negligible amount of HCHO present in the atmosphere.

The cascade impactor provided solid, reproducible particle size data that indicated that the XM81 grenade detonation produced considerable respirable fragments and particles (<10 μm). The fiber exposure MMADs (except the mid concentration) and the geometric standard deviations were in close agreement, indicating the rats inhaled a similar particle size during each exposure.

Direct comparison tc the SEM particle sizing is not possible. Generally, both methods indicate that the majority of fuse/fuel particles are twofold smaller than the fiber fragments. Comparing the fuse/fuel SEM data with those from the loaded grenades suggests that the majority of particles below 0.3 μm are by-products of the combustion materials, while the majority above 0.3 μm result from carbon fibers. Everything above 0.7 μm came

from the fibers. In all cases, every particle observed under SEM can be considered respirable, and no particles were larger than 10 μm.[16]

SEM has been used to observe deposition of particles in the lungs of rats and mice. Previous studies on asbestos, fiber glass, alpha-quartz, and volcanic ash have shown preferential deposition at the bifurcations of alveolar ducts over alveolar spaces and duct surfaces.[17] In this study, carbon fragments were identified in the trachea and bronchus, but none were in the alveolar regions, which makes it difficult to predict a deposition pattern. No toxic, inflammatory, degenative or proliferative changes other than the presence of a few macrophages were elicited by the presence of the pigment. The lesions in the eye may be treatment related. Residual material may have collected and elicited a minimal to mild inflammatory reaction in the eyes of the rats exposed to carbon fibers in this study. Because carbon fibers are known to produce an irritant response,[8] eye protection is recommended.

5. CONCLUSIONS

Inhalable fiber fragments were produced from the XM81 grenade detonation. Scanning electron microscopy indicated the fragments were deposited primarily in the trachea and bronchus. Only smaller sized particles reached the lungs and were visible as black/brown pigment contained in the alveolar macrophages.

Short term, high-level exposures of these fragments did not appear to be harmful to the rats exposed in this study. There were no adverse pulmonary effects observed through the 3 months post-exposure period. A mild inflammatory reaction developed in the eyes of the carbon fiber exposed rats. It is recommended that eye and skin protection be utilized to prevent an irritant response from exposure to carbon fibers.

LITERATURE CITED

1. Dahlquist, B.H., Evaluation of Health Aspects of Carbon Fibers, Master of Science Thesis, University of North Carolina, Chapel Hill, NC, 1984.

2. Gross, P., and Braun, D.C., Toxic and Biomedical Effects of Fibers, Noyes Publisher, Park Ridge, NJ, 1984.

3. Donnet, J.B., and Bansal, R.C., Carbon Fibers, Marcel Dekker Inc., New York, NY, 1984.

4. Gross, P., "Consideration of the Aerodynamic Equivalent Diameter of Respirable Mineral Fibers," Am. Ind. Hyg. Assoc. J. Vol. 42, pp 449-452 (1981).

5. Trimbell, V., "Deposition and Retention of Fibers in the Human Lung," Ann. Occup. Hyg. Vol. 26, pp 347-369 (1982).

6. Trimbell, V., "The Inhalation of Fibrous Dusts," Ann. N.Y. Acad. Sci. Vol. 132, pp 255-273 (1985).

7. Thomson, S.A., Hilaski, R.J., Wright, R., and Mattie, D., Nonrespirability of Carbon Fibers in Rats from Repeated Inhalation Exposure, CRDEC-TR-149, U.S. Army Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD, September 1990, UNCLASSIFIED Report (AD A228 196).

8. Thomson, S.A., "Toxicology of Carbon Fibers," Appl. Ind. Hyg. Special Ed. (1989).

9. Vu, V.T., Health Hazard Assessment of Nonasbestos Fibers, Office of Toxic Substances, U.S. Environmental Protection Agency, Washington, DC, 1988.

10. Smith, L.H., Carbon Fibers Health Effects, Oak Ridge National Laboratory, Oak Ridge, TN, 1986

11. Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, National Institute of Health, Washington, DC, 1985.

12. Deskin, R., Mosbergh, A., McNeill, K., Frazier, M., Pelroy, D., and Fisher, G., Tier I Generation and Characterization of Three Microcrystalline Waxes, Acrawax C, MP-12, Mekon White and Tier II Bacterial Mutagenicity and Mammalian Cell Cytoxicity Evaluation of the Collected Particulate Samples, CRDEC-CR-85037, U.S. Army Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD, July 1985, UNCLASSIFIED Report (AD B094 907L).

13. Thomson, S.A., Burnett, D.C., Crouse, C.L., Hilaski, R.J., and Muse, W.T., Acute Inhalation Toxicity of Pyrotechnically Disseminated Terephthalic Acid, CRDEC-TR-88134, U.S. Army Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD, July 1988, UNCLASSIFIED Report (AD A199 234).

14. Hanks, J.H., and Wallace, J.H., "Determination of Cell Viability," Proc. Soc. Exp. Biol. Med. Vol. 98, pp 188-192 (1985).

15. Gad, S.C., and Weil, C.S., "Statistics for Toxicologists," In Principles and Methods of Toxicology, pp 273-320, A.W. Hayes, Raven Press, New York, NY, 1984.

16. Petersen, E.R., and Astarita, J.L., Distribution of Respirable Particles From Detonation of XM81 Grenades Loaded With Pre-Cut Graphite Fibers, CRDEC-TR-336, U.S. Army Chemical Research, Development and Engineering Center, Aberdeen Proving Ground, MD, March 1992, UNCLASSIFIED Report (AD B164 059L).

17. Brody, A.R., and Roe, M.W., "Deposition Pattern of inorganic Particles at Alveolar Level in the Lungs of Rats and Mice," Am. Rev. Respir. Dis. Vol. 128, pp 724-729 (1983).

APPENDIX A

XM81 GRENADE COMPONENTS

1. EXPULSION CHARGE (Propellant)

   - Class 6 Black Powder (Sulphur, Carbon (Charcoal), Potassium Nitrate)
     MIL-P-223 (Black, Powder, Class 6)

2. DELAY DETONATOR ASSEMBLY

   - Composition A-1A (Potassium Nitrate, Sodium Nitrate, Charcoal, Sulphur)
     MIL-P-22264 (Powder, Ignition, Gasless A-1A)

   - Lead Azide

     MIL-L-46225 (Lead Azide, RD-1333)

   - RDX (Cyclotrimethylenetrinitramine)
     MIL-R-398 (RDX, Type B)

   - Zirconium/Nickel Delay (Nickel, Zirconium, Barium Chromate, Potassium Perchlorate)
     MIL-C-13739 (Composition Delay Type III)

3. TRANSFER LEAD

   - Plastic Bonded Explosive (PBX), Type I

   - HMX (Cyclotetramethylenetetranitramine), copolymer of vinylidene fluoride and hexafluoropropylene
     MIL-E-81111 (Type I, Class 3)

4. BOOSTER LEAD

   - Composition CH-6 (RDX, Calcium Stearate, Graphite)
     MIL-C-21723

5. BURSTER CHARGE

   - Detonation Cord, 240 grains per foot
     HMX, (Cyclotetramethylenetetranitramine)

6. CARBON FIBERS AND BRASS FLAKES

Blank

APPENDIX B

ANIMAL HEALTH

DEPARTMENT OF THE ARMY
U.S. ARMY CHEMICAL RESEARCH, DEVELOPMENT AND ENGINEERING CENTER
ABERDEEN PROVING GROUND, MARYLAND 21010-5423

REPLY TO ATTENTION OF

SMCCR-RST-V

15 October 1991

MEMORANDUM FOR Dr. Thomson

SUBJECT: Inspection of Animal Shipment

Dr. Thomson:

On 20 September 1991 I examined the shipment of 216 male F344 rats from Charles River for your Protocol number 22090000A269 that arrived on 18 September 1991. All animals appeared normal. Cultures of tracheal washes and gastrointestinal tract were performed by USAMRICD. No growth was found on tracheal wash cultures and no enteric pathogens were isolated from the gastrointestinal tracts.

These animals meet the criteria for utilization on your protocol.

BILLY W. HOWARD
MAJ, VC
Chief, Veterinary
Services Branch

APPENDIX C

SEM TISSUE EXAMINATION

PROTOCOL CRDEC 1216222A552

P.I. Dr. Sandra Thomson

INTERIM REPORT #2 22 January 1992

SCANNING ELECTRON MICROSCOPY IMPRESSIONS

We have searched for carbon particulates as depicted in the grenade sample (Test C grenade) in the trachea, carina, primary bronchi and alveoli of exposed rats. The following table summarizes our finding at this juncture (18 animals).

		0hr	24hr	14day
CONTROL:				
	Trachea	-	-	-
	Bronchus	-	-	-
	Carina	-	n/a	-
	Alveolus	-	-	-
	Animals	1	1	1
FUSE/FUEL:				
	Trachea	+(1,39)	-	+(1,94)
	Bronchus	+(1,39)	-	-
	Carina	-	-	-
	Alveolus	-	-	-
	Animals	2	2	2
HIGH CONC:				
	Trachea	-	+(3,12)	+(1,119)
	Bronchus	+(2,40)	+ (1,168)	-
	Carina	-	-	-
	Alveolus	-	-	-
	Animals	3	3	3

NOTES: " ANIMALS" refers to the number of animals investigated

" (3, 12)" First number refers to numbers of carbon particulates found
Second number refers to the ANIMAL EXPERIMENTAL NUMBER.

Although we are indicating that the CONTROLS, FUSE/FUEL ANIMALS and HIGH CONCENTRATION were free of specific carbon particulates, we did find other particulates that we consider as incidental to animal handling and environmental conditions (hair, intubation sutures, unidentified debris). In some cases this was in significant amounts. We plan to extend the study to include x-ray microanalytical identification of some of the particulates. A new light element detector is presently being installed on our scanner for this kind of analysis.

Please consider this an interim report. Things may change as we look longer.

John Petrali, Phd
Team Leader

P R O T O C O L C R D E C 1 2 1 6 2 2 2 A 5 5 2

P.I. Dr. Sandra Thomson

INTERIM REPORT #3 7 February 1992

SCANNING ELECTRON MICROSCOPY IMPRESSIONS

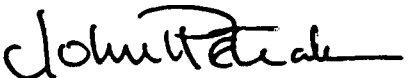
We have searched for carbon particulates as depicted in the grenade sample (Test C grenade) in the trachea, carina, primary bronchi and alveoli of exposed rats. Earlier tables summarized our findings at 0hr, 24hr, 14day. This table summarizes our findings at the 3 month time period. We have now completed the scanning imaging portion of the study (24 animals).

CONTROL:	3 MONTHS
Trachea	-
Bronchus	-
Carina	-
Alveolus	-
Animals	1

FUSE/FUEL:	
Trachea	-
Bronchus	-
Carina	-
Alveolus	-
Animals	2

HIGH CONC:	
Trachea	+ *(10,148) (1,20)*
Bronchus	-
Carina	-
Alveolus	-
Animals	3

NOTES: In addition to particulate imaging we also found a large proliferation of inflammatory cells, probably macrophages, on the mucosal surface of the trachea of all high concentration animals at the 3 month period. This probably represents a response to a persistent presence of particulates or other irritants. We plan to extend the study to add x-ray microanalysis of some of the high concentration particulates.



John Petrali, Ph.D
Team Leader

Roger,

This is to inform you of the procedures used in the x-ray microanalysis of your samples.

Selected specimens and experimental time frames were analyzed with a DELTA CLASS III KEVEX FISONS MICROANALYZER. Tracheal specimens were from:

Zero hour: Animal # (indicated on prints we sent you)

24 hour: Animal # (ditto)

3 months: Animal # (ditto)

Scanning Electron Microscope: Armay 1820.
Scope parameters: Accelerating Voltage 10Kv (see prints)
Working distance 15 millimeters
Magnification (see prints)
Specimen Tilt 25 degrees
Horizontal Distance of the Detector 5 centimeters

X-ray spectra were acquired for 100 seconds for each particulate. Carbon peaks were acquired at 0.282 KeV. Silica peaks were acquired at 1.740 KeV.
Carbon and silica peaks were routinely processed as follows:

Peaks automatically identified
Escape peaks removed
Background subtracted

Our analyses was qualitative. No quantitation was attempted.

This is the final report you will receive from us. We consider the experiment completed at this end. We have no wish to publish the morphological / analytical data independent of CRDEC. We will keep microscopic prints / x-ray data in our files for awhile. We will be using portions of the study for ICD SHOW TOURS that occur at regular intervals here.

If you have any questions in my absence please be in contact with Ken Mills. He performed most of the analysis.

John P. Petrali, PhD
15 May 1992

Roger,

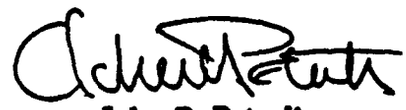
Attached are x-ray spectra with scanning micrographs of selected particulates. All particulates are from the trachea of animals as follows:

- All are high concentration animals
- Zero hour, 24hour and 3 months

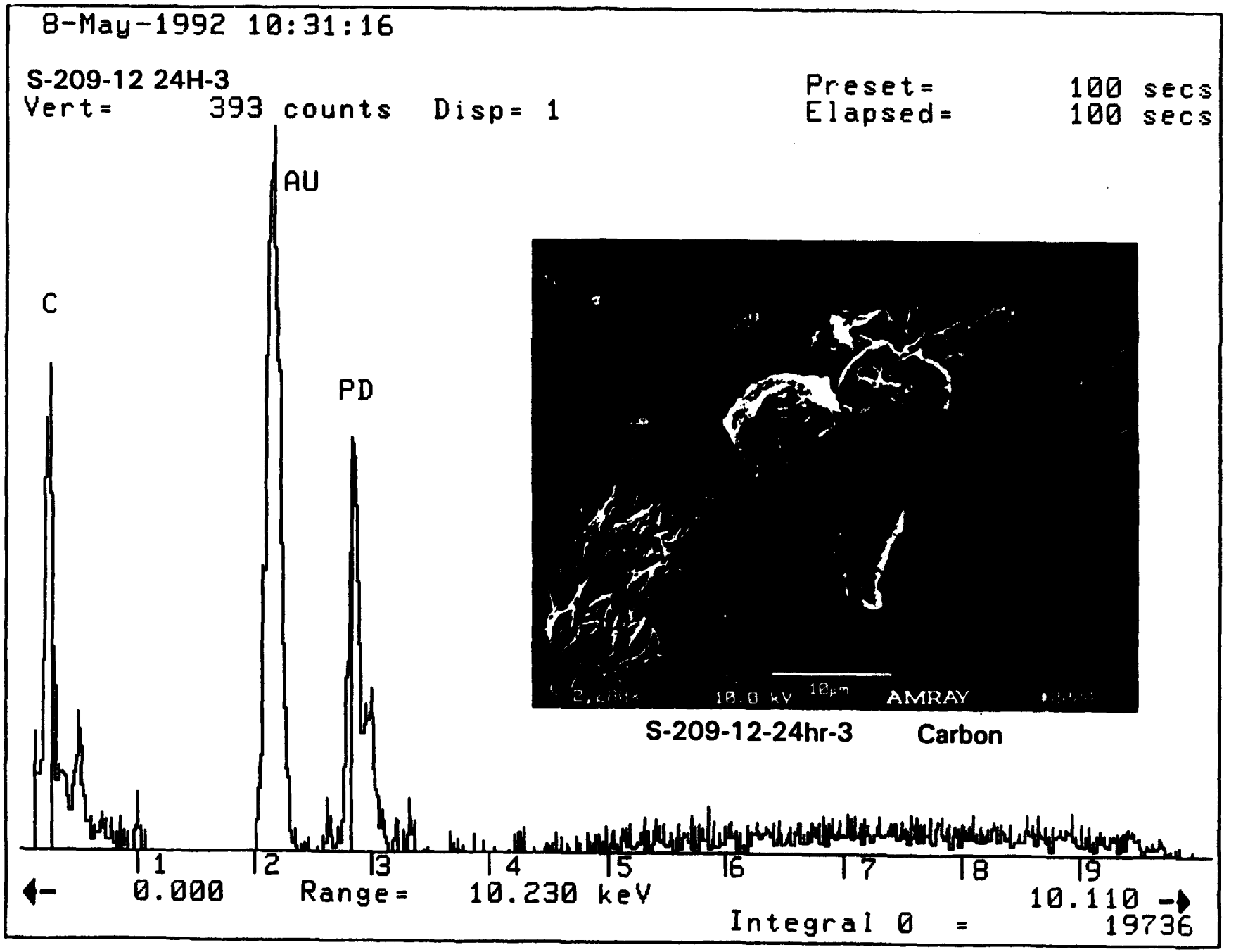
The spectra are indentifying as major peaks, carbon, silica, gold, palladium, oxygen. Gold and palladium are our specimen coating metals and are present in all specimens. The particulate most prevalent at zero hour was carbon. The particulate most prevalent at 3 months was silica. This conclusion is strictly qualitative.

We will send more complete analysis parameters at a later time.

We hope this information is useful to you.



John P. Petrali
12 May 1992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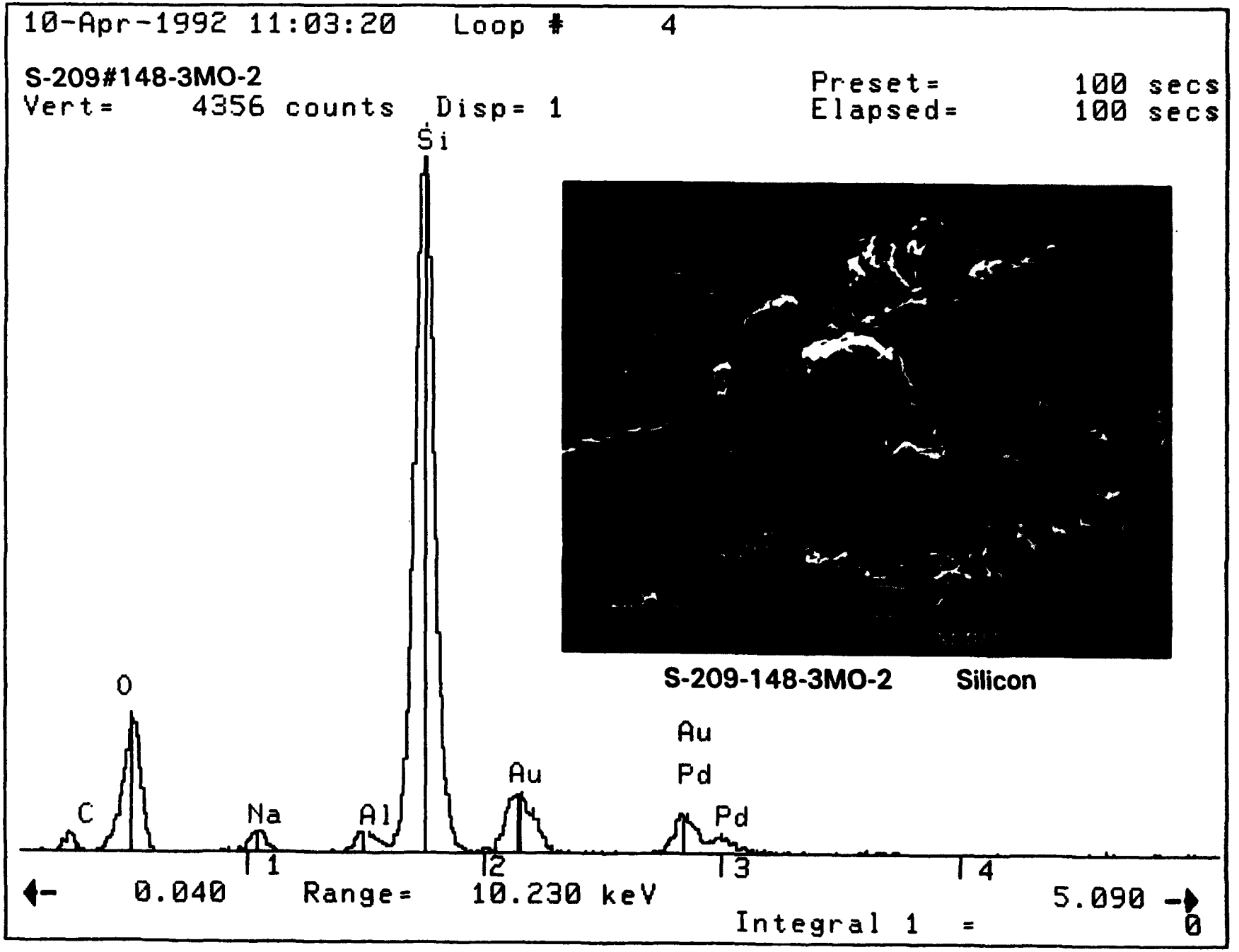

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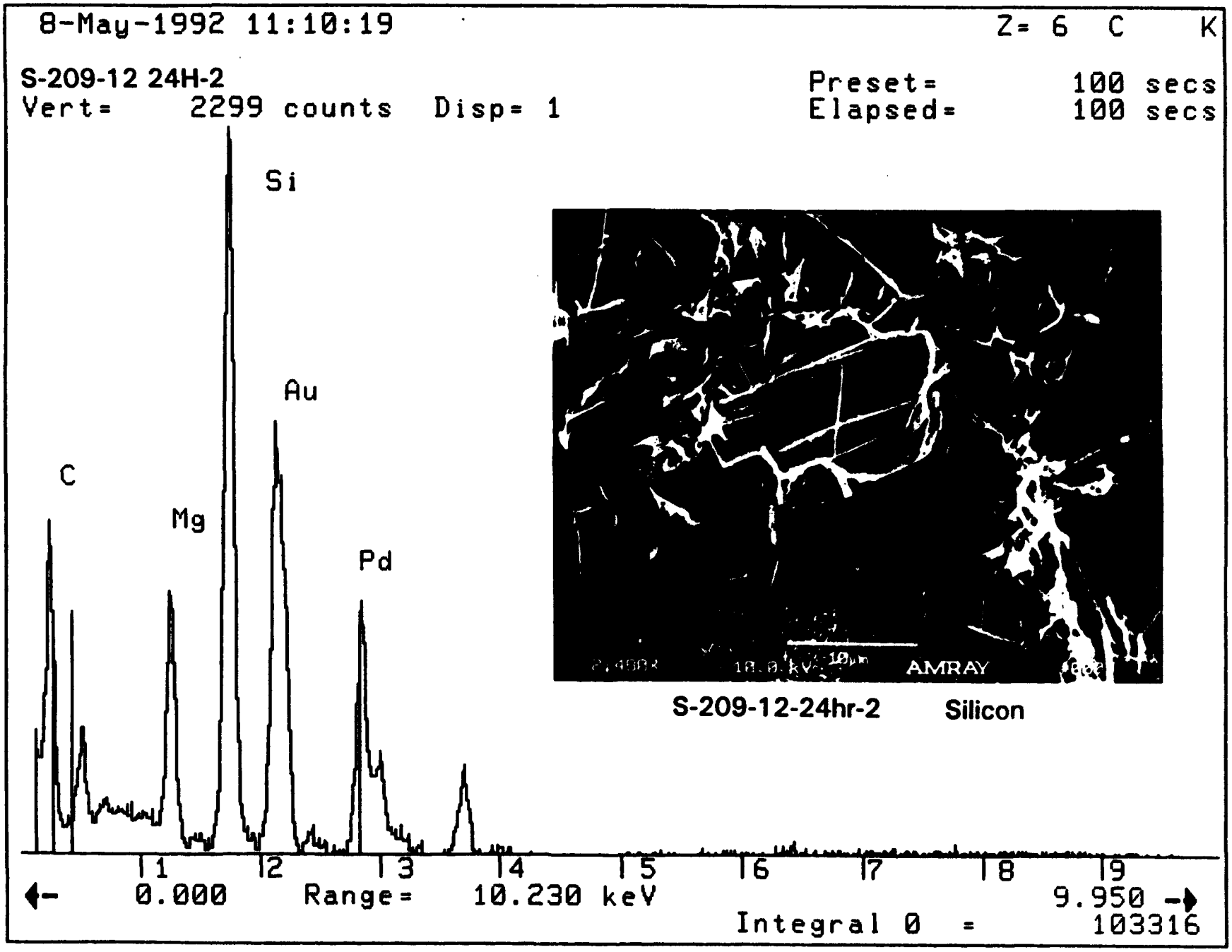

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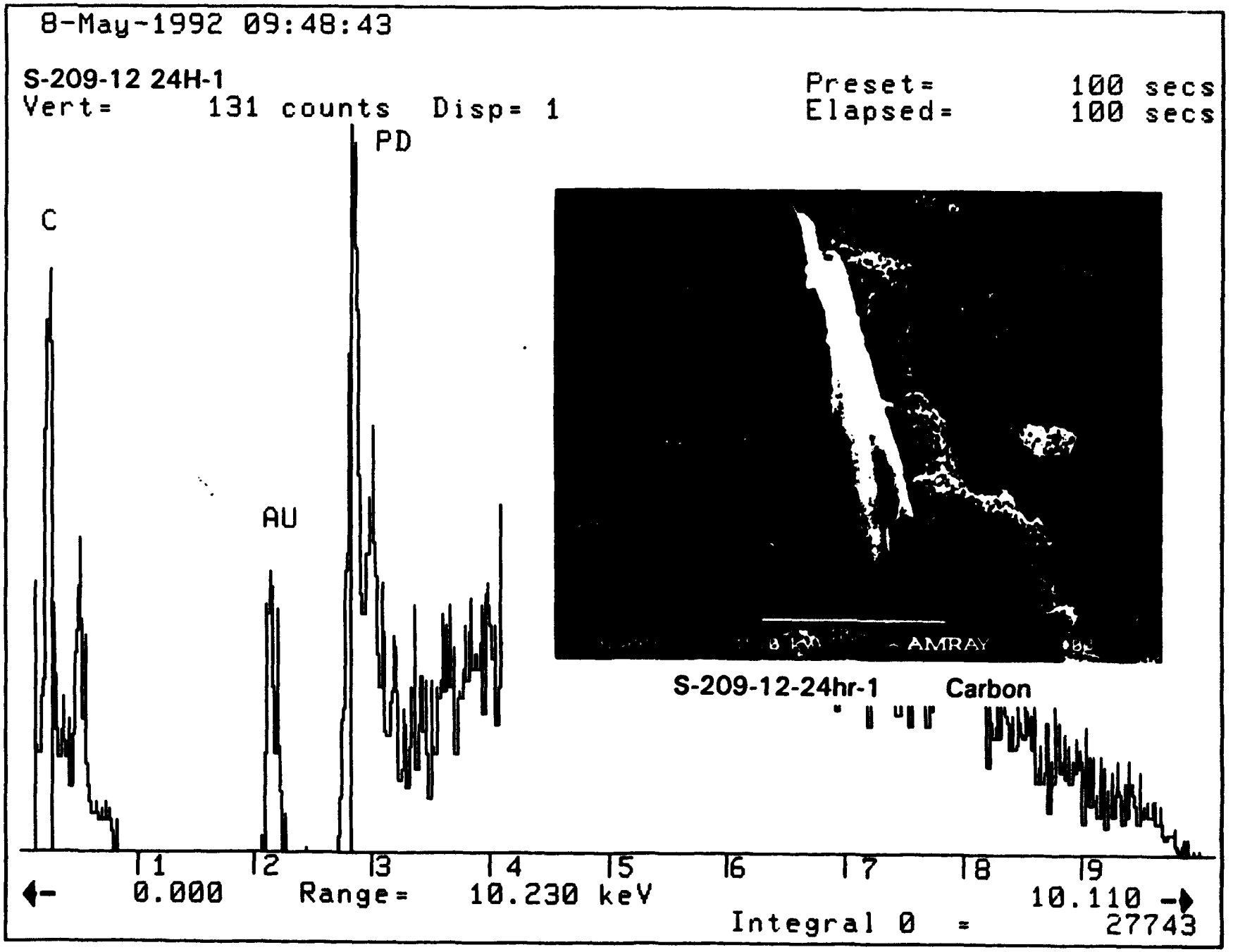
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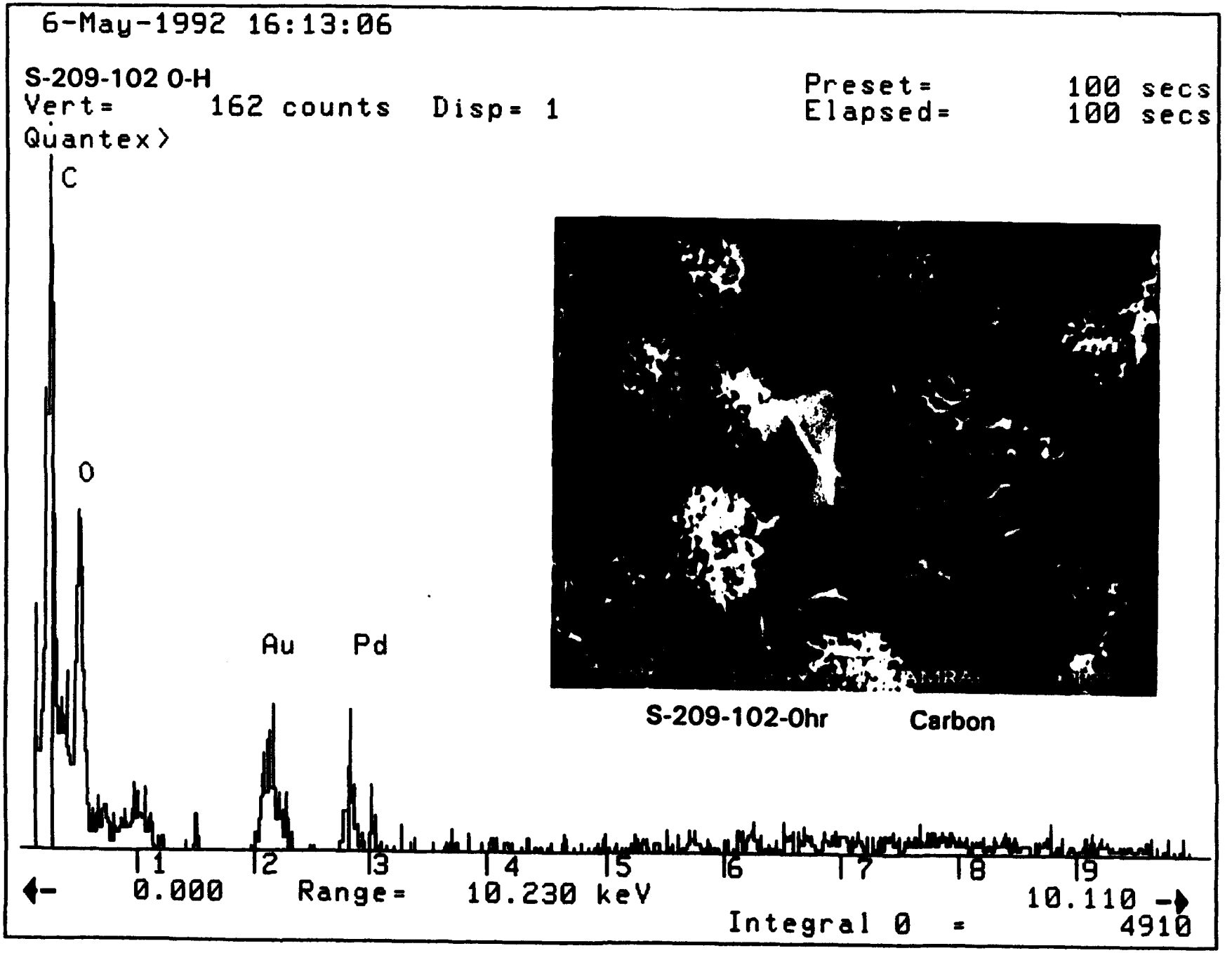

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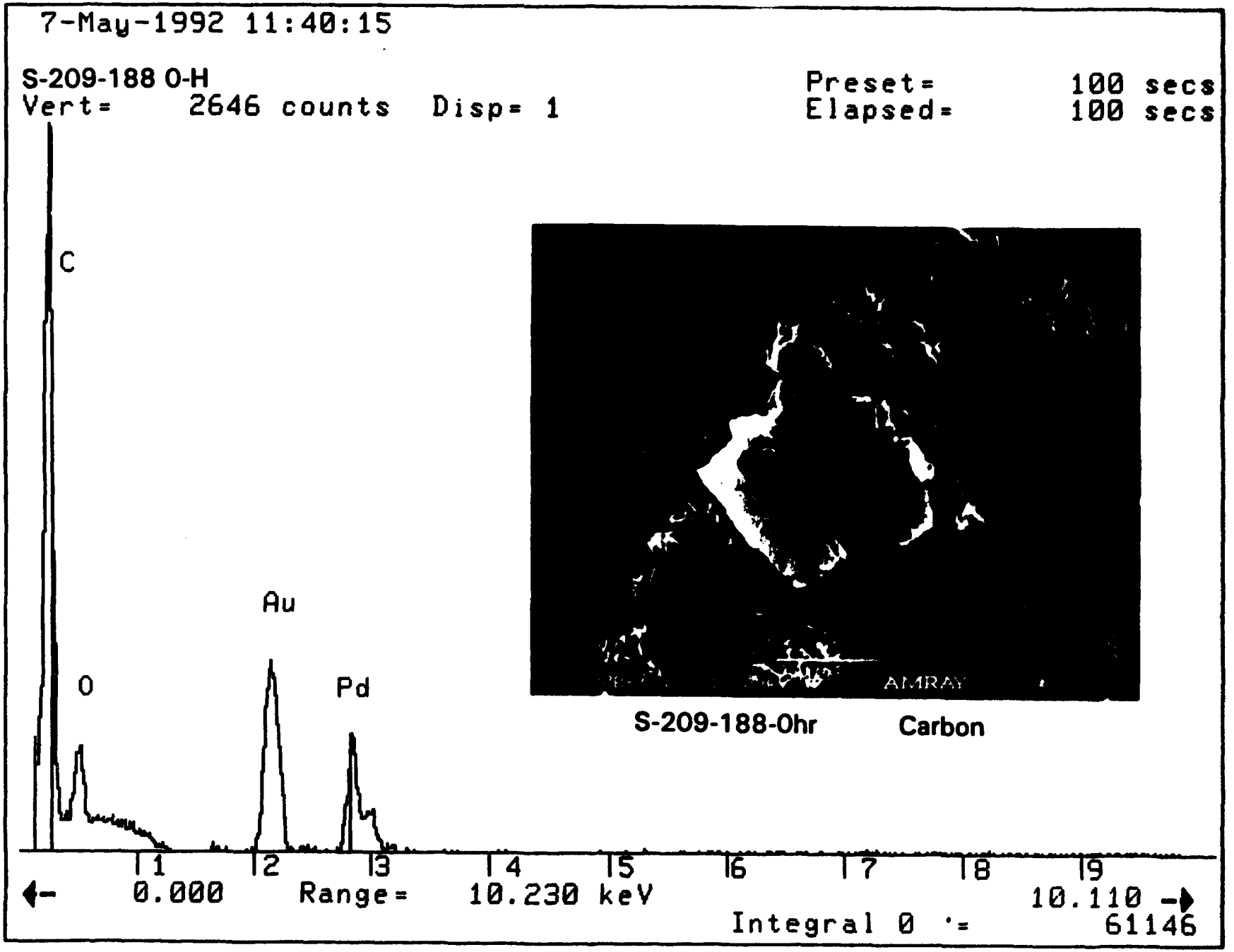

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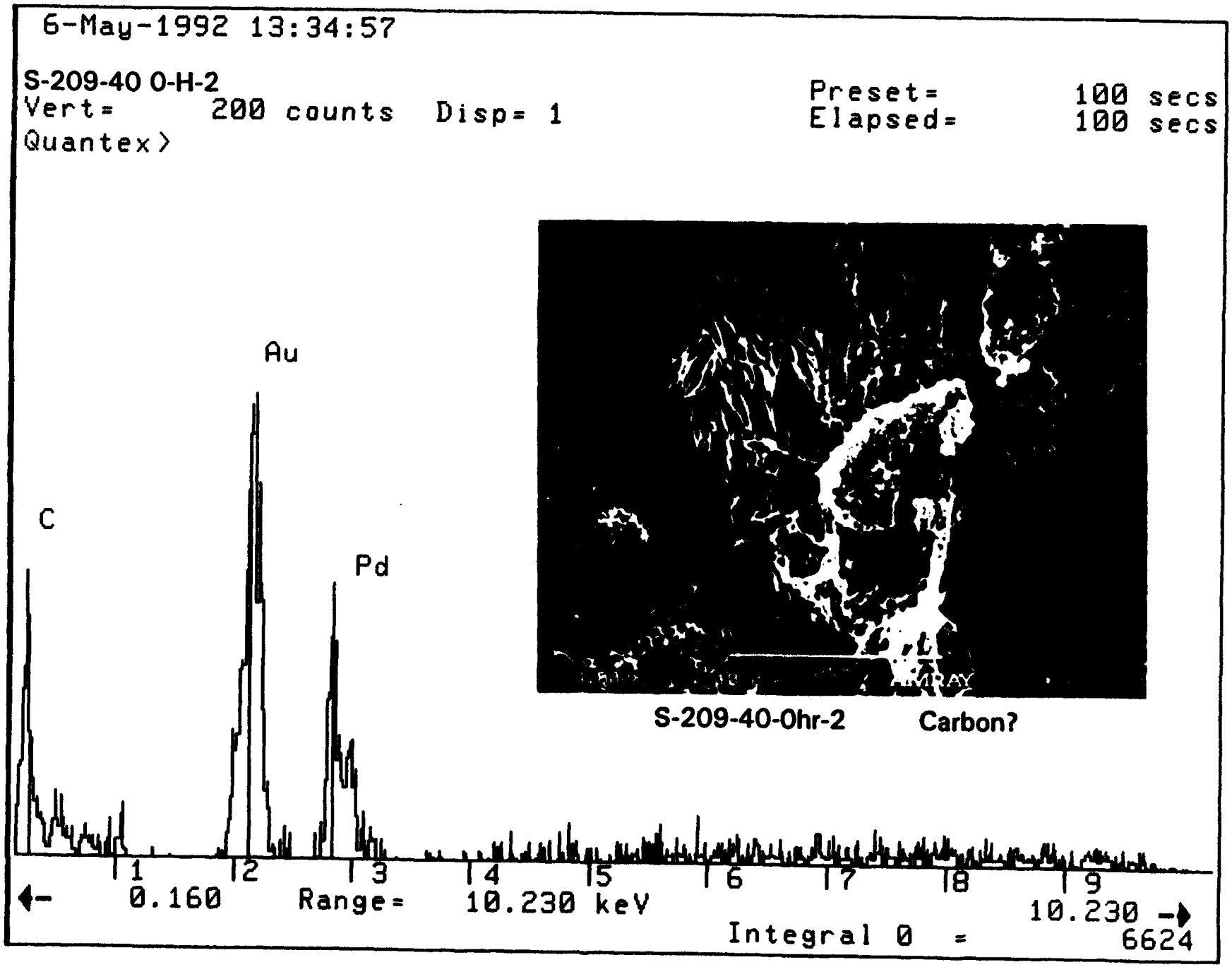
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7
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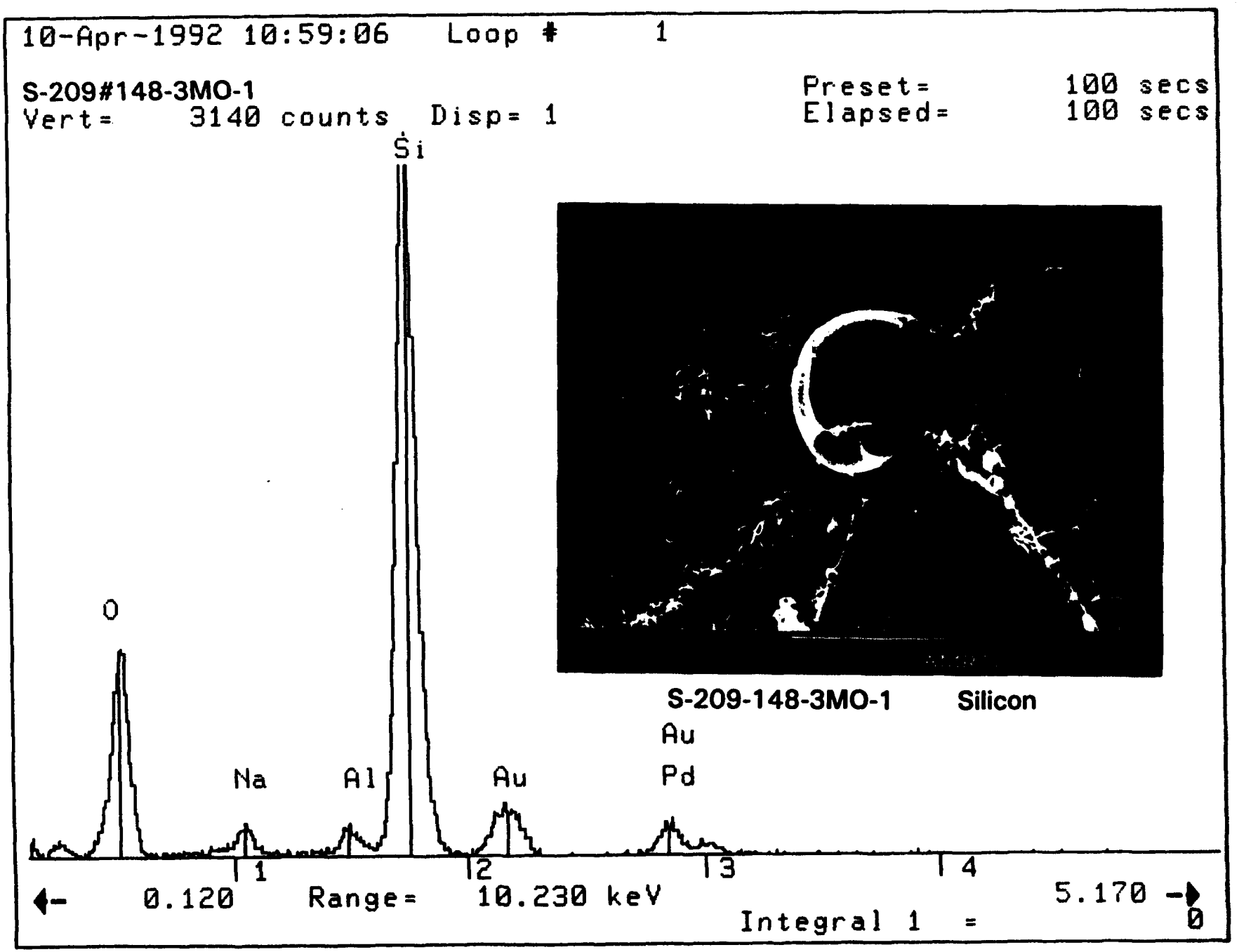

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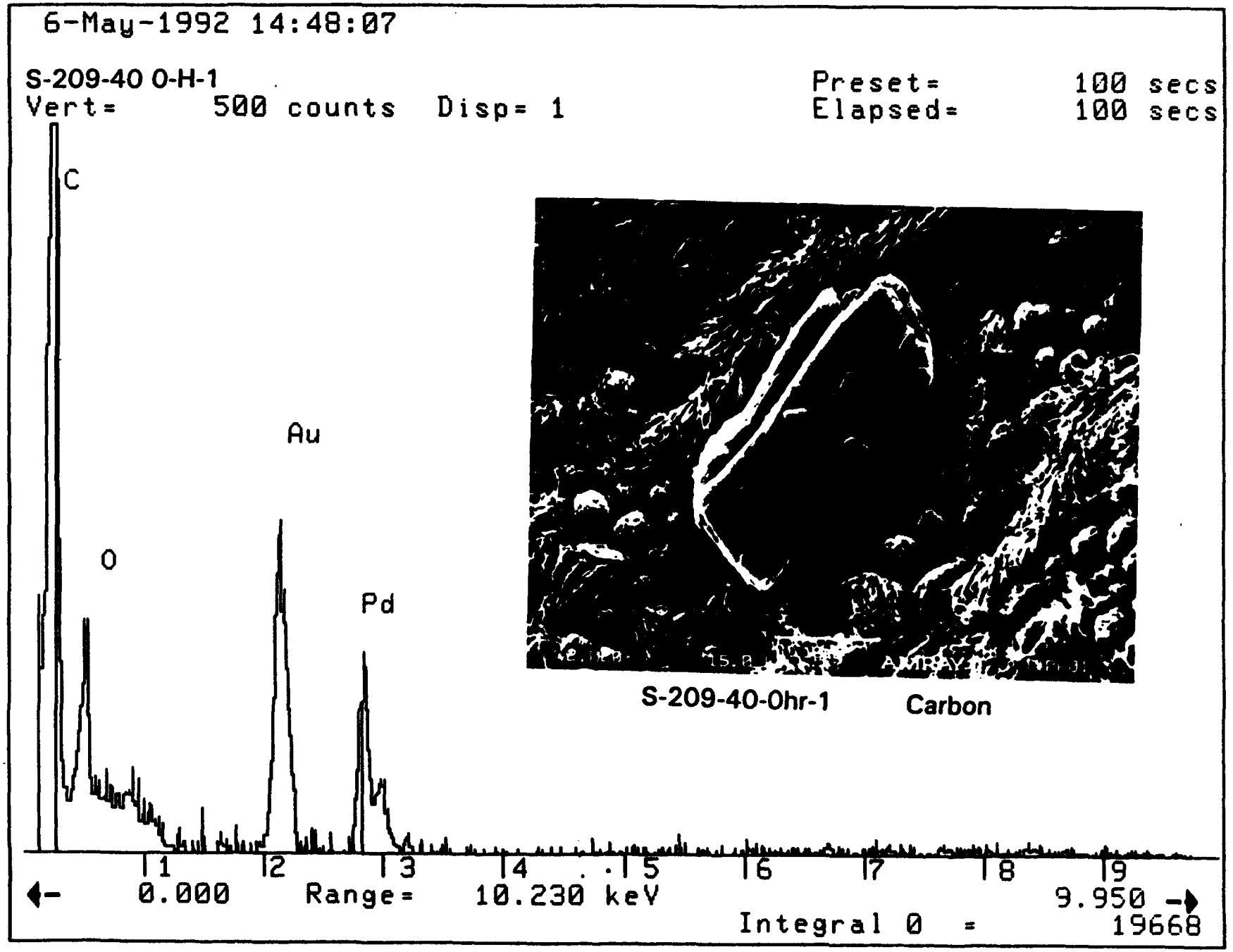

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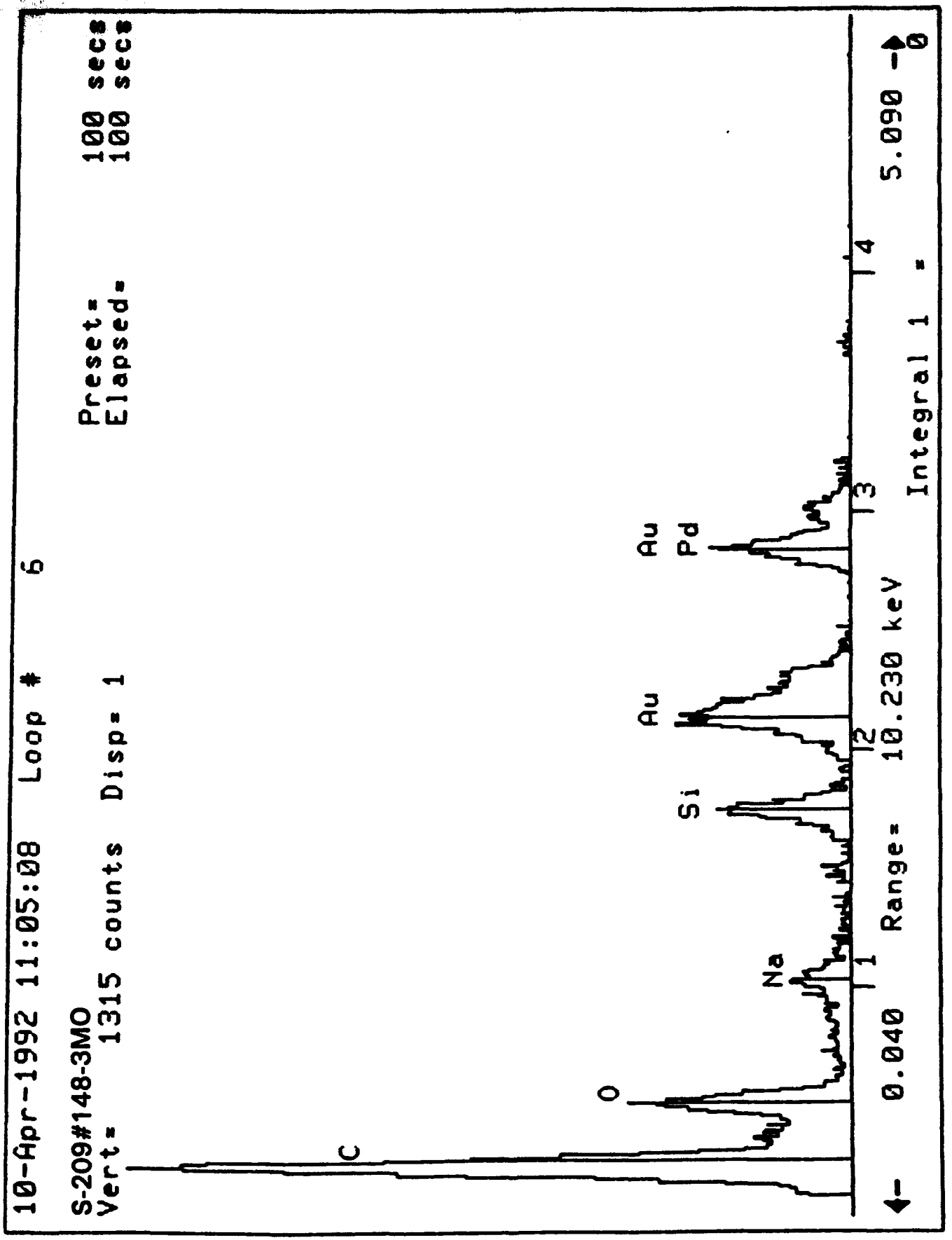
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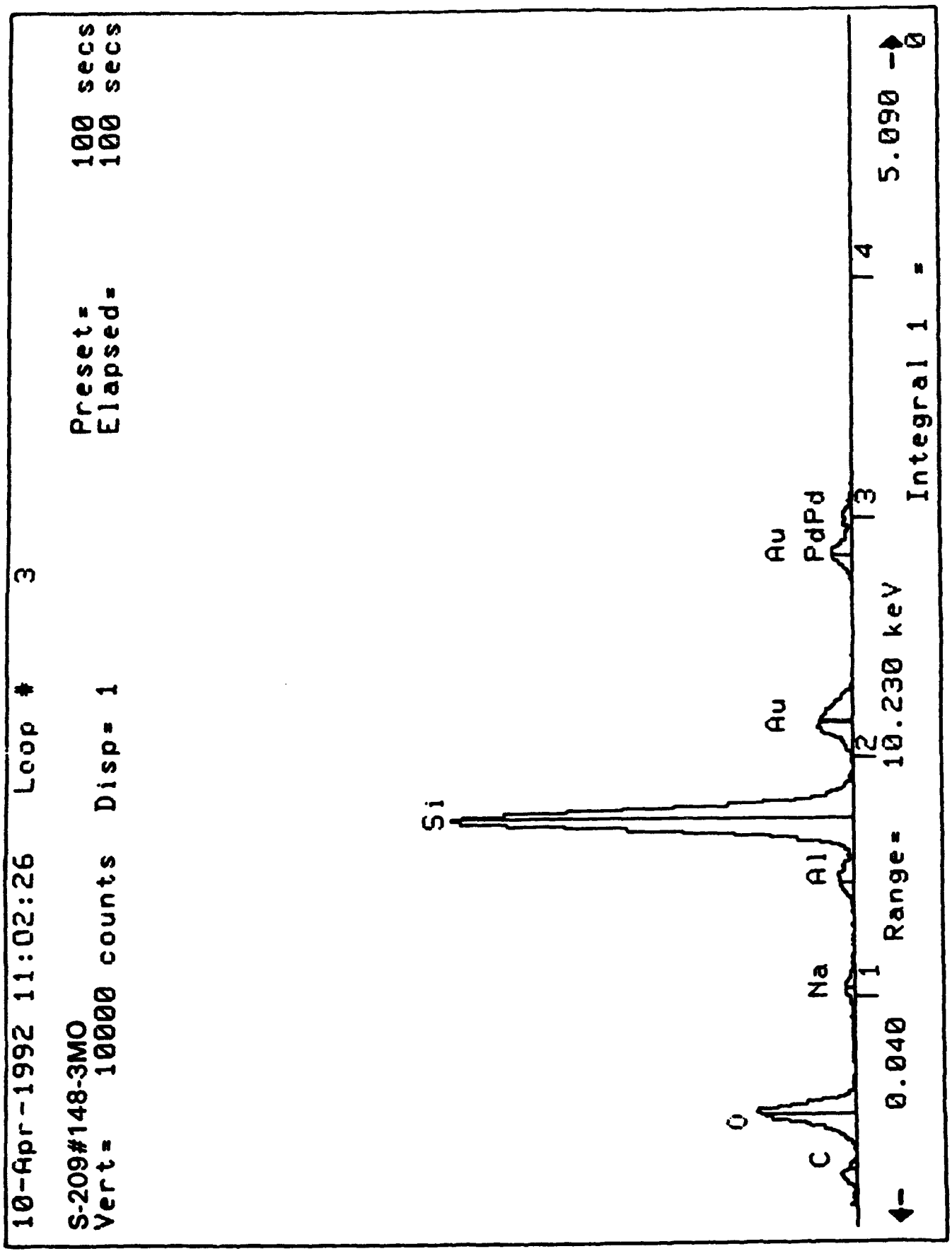
Si
Au
Au
Pd
1
2
3
4
0.040 Range= 10.230 keV 5.090
Integral 1 =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2
3
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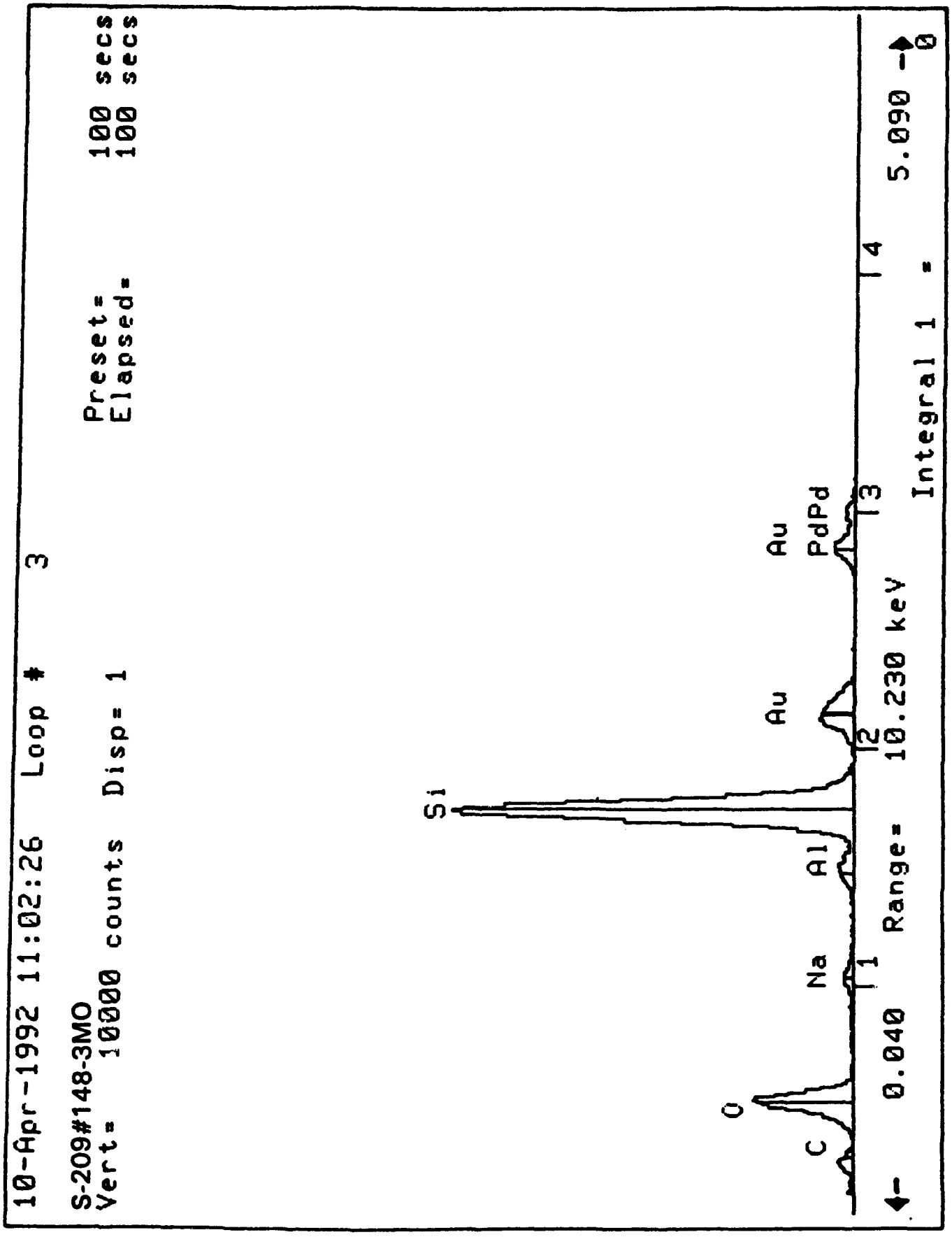
0.040 Range= 10.230 keV 5.090
0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O
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Al
Si
Au
Au
PdPd
1
2
3
4
0.040 Range= 10.230 keV Integral 1 = 5.090
0

Blank

APPENDIX D

HISTOPATHOLOGY SUMMARY

PATHOLOGY REPORT

Acute Inhalation Toxicity Effects of Explosively Disseminated Carbon Fibers (XM81 Grenade) 24-Hour and 14-Day Sacrifices

Protocol No. 22091000A269

INTRODUCTION

This report prepared by Pathology Associates, Inc. (PAI) for the Toxicology Division, Research Directorate, Chemical Research Development and Engineering Center (CRDEC), Aberdeen Proving Ground, MD 21010-5423, presents the results of gross and microscopic examination of tissues from rats exposed via inhalation to various concentrations of explosively disseminated carbon fibers from the XM81 Grenade and sacrificed 24 hours or 14 days post-exposure (PE) in accordance with Protocol No. 22091000A269. The pathology evaluation was conducted under the provisions of Contract No. DAAA15-91-M-0081.

EXPERIMENTAL DESIGN AND METHODS

Male Fischer 344 rats were exposed by whole-body inhalation to one of three concentrations of carbon fibers (XM81 Grenade) for 30 minutes. Air-exposed and fuse-fuel-exposed rats served as controls. Twenty-four hours or fourteen days PE the rats were euthanized via asphyxiation with carbon dioxide and necropsied in accordance with the provisions of PAI SOPs and contract requirements. The experimental design for animals designated for pathologic evaluation is as follows:

EXPERIMENTAL DESIGN

DOSE GROUP	NUMBER OF ANIMALS	
	24 Hr. (PE) Sacrifice	14 Day (PE)Sacrifice
Air-exposed Control	6	6
Fuse/Fuel-exposed Control	6	6
Carbon Fibers (Low Concen.)	6	6
Carbon Fibers (Medium Concen.)	6	6
Carbon Fibers (High Concen.)	6	6

Tissues required by contract were fixed in 10% neutral buffered formalin for at least 48 hours. For the air-exposed control and high dose groups, all tissues required by contract (full histopathology) were processed through paraffin, sectioned, at approximately 6 microns, stained with hematoxylin and eosin, and examined

microscopically. For the remainder of the groups, as required by contract, limited tissues consisting of lung, trachea, tracheobronchial lymph node(s) (when present in section), liver, kidneys, nose (4X) and gross lesions were prepared and evaluated.

RESULTS

Gross Pathology

There were no treatment-related gross lesions noted in the rats necropsied at 24 hours PE or 14 days PE.

Histopathology

Microscopic findings are presented in tabular format in Sections II and III. Summary data are presented by dose group in the Project Summary Tables, while individual diagnoses are presented in the Tabulated Animal Data Tables. The Reports Code Table (Appendix 1) defines the symbols for distribution and severity used in the tables. Abbreviations used in any of the tables are explained in Appendix 2.

The administration of explosively disseminated carbon fibers resulted in the deposition of brown-black pigment in the alveolar macrophages of exposed rats. The presence of the pigment did not result in any inflammatory reactions other than phagocytosis of the pigment by the alveolar macrophages. Alveolar macrophages containing the black pigment were also present in the lungs of some of the fuse/fuel-exposed control group.

24-Hour Sacrifice

In all of the carbon fiber (high concentration)-exposed rats necropsied at 24 hours PE, brown-black pigment was present within alveolar macrophages randomly scattered throughout the lung. This pigment elicited no inflammatory reaction other than phagocytosis of the pigment by the alveolar macrophages. The pigment-containing macrophages were also present in four of six rats in the low concentration exposure group and in six of six rats in the medium concentration exposure group. The number of pigment-containing macrophages increased from the low to high dose group, however, few such macrophages were present even in the high concentration exposure group. A very small number of pigment-containing macrophages were also present in the lungs of one rat in the fuse/fuel-exposed group. The amount of pigment in these macrophages was extremely small.

14-Day Sacrifice

As in those animals necropsied at 24 hours PE, the lungs of all of the carbon fiber (high concentration)-exposed rats necropsied at 14 days PE contained brown-black pigment present within alveolar macrophages randomly scattered throughout the lung. The pigment-containing macrophages were also present in all rats in the low and medium concentration exposure groups. The severity and distribution of the response

was the same as that seen in corresponding groups necropsied at 24 hours PE. A very small number of pigment-containing macrophages were also present in the lungs of four rats in the fuse/fuel-exposed group. The amount of pigment in these macrophages was extremely small.

Miscellaneous

Several incidental lesions were present in the rats necropsied at 24 hours PE and 14 days PE. Only two of these warrant further comment. Multifocal, centrilobular, hepatocellular cytoplasmic vacuolization was noted in many of the rats. This change probably reflected the presence of glycogen and was not considered a significant pathologic change.

Focal granulomatous inflammation was noted in the bulbar conjunctiva of the eye of two high dose and two air-exposed control animals necropsied at 24 hours PE. The lesions were of minimal severity and not considered a significant pathologic change. The lesions were not observed in any animals necropsied at 14 days PE.

CONCLUSION

Male Fischer 344 rats were exposed by inhalation to one of three concentrations of explosively disseminated carbon fibers for 30 minutes. Air-exposed and fuse/fuel-exposed rats served as controls. Twenty-four hours or fourteen days PE, the rats were euthanized via asphyxiation with carbon dioxide and necropsied.

Under the conditions of this work, the only significant treatment-related finding was the presence of brown-black pigment within alveolar macrophages of rats exposed to both carbon fibers and fuse/fuel necropsied at 24 hours or 14 days PE. No other significant morphologic changes were present.

Lynda L. Pippin, DVM
February 16, 1992

PATHOLOGY REPORT

Acute Inhalation Toxicity Effects of Explosively Disseminated Carbon Fibers (XM81 Grenade) Three-month Sacrifice

Protocol No. 22091000A269

INTRODUCTION

This report by Pathology Associates, Inc. (PAI) for Toxicology Division, Research Directorate, Chemical Research Development and Engineering Center (CRDEC), Aberdeen Proving Ground, MD 21010-5423, presents the results of gross and microscopic examination of tissues from rats exposed via inhalation to various concentrations of explosively disseminated carbon fibers, and sacrificed three months post-exposure (PE) in accordance with Protocol No. 22091000A269. The pathology evaluation was conducted under the provisions of Contract No. DAAA15-91-M-0081.

EXPERIMENTAL DESIGN AND METHODS

Male Fischer 344 rats (six per group) were exposed by whole-body inhalation to one of three concentrations of carbon fibers for 30 minutes. Air-exposed and fuse/fuel-exposed rats served as controls. Twenty-four hours, 14 days, or three months PE the rats were euthanized via asphyxiation with carbon dioxide and necropsied in accordance with PAI SOPs and contract requirements. The draft pathology report for the first two necropsies (24 hours and 14 days) has been previously submitted by Dr. Lynda Pippin.

Tissues required by contract were fixed in 10% neutral buffered formalin for at least 48 hours. For the Air-exposed Control and High dose groups, all tissues required by contract (full histopathology) were processed through paraffin, sectioned, at approximately 5 µm, stained with hematoxylin and eosin, and examined microscopically. For the remainder of the groups, as required by contract, limited tissues consisting of lung, trachea, tracheobronchial lymph node(s) (TBLN), liver, kidneys, nose (4X), and target tissues (identified in the high dose group) were prepared and evaluated.

RESULTS

Gross Necropsy Findings

No treatment-related lesions were noted at necropsy or during trimming of the tissues. Several incidental lesions were noted, however. These are as follows:

Air-exposed (Control)
1133 - Lymph node, mandibular: Enlarged (8 x 6 mm) (Detected at trim)
1143 - Lymph node, mandibular: Enlarged (6 x 5 mm) (Detected at trim)
1162 - Lymph node, mandibular: Enlarged (7 x 6 mm) (Detected at trim)
1178 - Lymph node, mandibular: Enlarged (8 x 4 mm) (Detected at trim)

Fuel/fuse
1026 - Liver, median lobe: Nodule, brown (10 x 10 x 10 mm) (hernia)

Low
1109 - Testes, bilateral: Small

High
1100 - Lymph node, mandibular: Enlarged (8 x 6 mm) (Detected at trim)

Histopathology

Microscopic findings are presented in tabular format in Sections II and III. Summary data are presented by dose group in the Project Summary Tables, while individual diagnoses are presented in the Tabulated Animal Data Tables. The Reports Code Table (Appendix 1) defines the symbols for distribution and severity used in the tables. Abbreviations used in any of the tables are explained in Appendix 2. The sites "Nose 1, 2, 3, and 4" in this report are the same as "Nose, 1, 3, 5, and 6," respectively, in the 24-hour/14-day report. The tissue "sternum" in this report represents bone marrow. Correlations of gross and microscopic findings are presented in Section IV.

The administration of explosively disseminated carbon fibers resulted in the deposition of brown/black granular pigment in the lungs of 5/6, 5/6, and 2/6 rats in the high, mid, and low dose groups, respectively. The pigment was contained within macrophages located in alveoli or terminal bronchioles. Very few pigment-laden macrophages were present, and only one or two particles of phagocytized pigment ($\leq 2\mu m$) were present in affected macrophages. No toxic or inflammatory reactions other than phagocytosis of the carbon fibers in the lung were present. A very small number of pigmented macrophages were present in the TBLN of 2/6, 1/5, and 2/5 rats in the high, mid, and low dose groups, respectively. No treatment-related changes were present in the lungs or TBLN of air-exposed or fuse/fuel-expose rats.

Small foci of granulomatous inflammation, were present within and/or just beneath the epithelial surface of the eye in the area of the bulbar conjunctiva, near the filtration angle and junction of the palpebral conjunctiva, in 4/6 rats in the high dose group. This change was not present in any air-exposed control rats (0/5). The lesion was characterized by the presence of mixed inflammatory cells and multinucleated giant cells surrounding amorphous, slightly basophilic material. In some affected rats, the inflammation appeared to be within or associated with lymphatics. In one high dose rat, the change was accompanied by mild epithelial dysplasia. The eyes of rats in the fuse/fuel, low, and mid dose groups were trimmed and examined for similar

changes. Similar lesions were present in the eyes of 2/6, and 1/5 rats in the low and mid dose groups, respectively. No lesions were present in the fuse/fuel-exposed rats.

All other microscopic lesions noted are considered to be incidental findings and will not be discussed further.

DISCUSSION

Pigment laden macrophages in affected rats were very few in number (less than one per 20 mid-power [200X] fields) and very difficult to identify due to the extremely small size and paucity of carbon fibers. No toxic, inflammatory, degenerative, or proliferative changes other than the presence of a few macrophages were elicited by the presence of the pigment. None of the changes in the other parts of the respiratory tract were determined to be treatment-related.

The lesions in the eye appear to be related to treatment. No carbon fibers were present within the inflammatory foci, however. The amorphous material within the multinucleated giant cells was not identified. Due to the apparent association with lymphatics in some of the affected eyes and the location of the lesions near the angle of the eye where the bulbar conjunctiva and palpebral conjunctiva meet, residual material may have collected and elicited a minimal to mild inflammatory reaction.

SUMMARY AND CONCLUSIONS

Male Fischer 344 rats were exposed by inhalation to one of three concentrations of explosively disseminated (XM81 Grenade) carbon fibers. Air-exposed and fuse/fuel-exposed rats served as controls. Three months PE the rats were euthanized via asphyxiation with carbon dioxide and necropsied.

No treatment-related gross lesions were present. Significant treatment-related microscopic findings consisted of the presence of small numbers of pigment-laden macrophages in the lungs of 5/6, 5/6, and 2/6 rats and in the TBLN of 2/6, 1/5, and 2/5 rats in the groups exposed to high, mid, and low doses of carbon fibers, respectively. In addition, small foci of granulomatous inflammation were present in the eyes of 4/6, 1/5, and 2/6 rats in the groups exposed to high mid, and low doses of carbon fibers, respectively. No treatment-related changes were present in the fuse/fuel-exposed group.

Lucas H. Brennecke, D.V.M.
Diplomate, ACVP
March 4, 1992

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APPENDIX E

MUTAGENICITY ASSAYS

STUDY TITLE
Salmonella Typhimurium/Microsome Reverse Mutation Assay

Project No.
ILS A052

Contract No.
DAAA15-91-D-0024

Test Article
Carbon Fibers

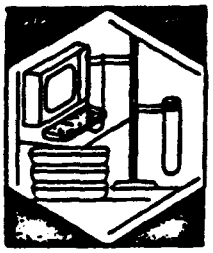
ILS Repository No.
91-40

Final Report Date
April 14, 1992

Sponsor
Mr. Fred K. Lee
U.S. Army Chemical Research Development, and Engineering Command
Aberdeen Proving Ground, MD 21005

Testing Facility
Integrated Laboratory Systems
801-8 Capitola Dr.
Durham, NC 27713

SUBMITTED BY



INTEGRATED LABORATORY SYSTEMS

PO Box 13501, Research Triangle Park, North Carolina 27709.

QUALITY ASSURANCE INSPECTION STATEMENT

ILS Project No.: A052
Test Article: Carbon fibers
ILS No.: 91-40
Study Title: Salmonella Typhimurium/Microsome Reverse Mutation Assay

Inspection/Audit	Date Performed	Date Reported to Study Director/Management
Phase Inspection:		
Culture inoculation - TA98	02/24/92	02/25/92;02/25/92
Plate Counts - TA1537	03/09/92	03/10/92;04/14/92
Data audit	04/02/92	04/08/92;04/13/92
Report audit	04/08/92	04/08/92;04/14/92

Kaye Cummings
Kaye Cummings, B.S.
Quality Assurance Officer

4/14/92
Date

CERTIFICATION OF GOOD LABORATORY PRACTICE

ILS Project No.: A052
Study Title: Salmonella Typhimurium/Microsome Reverse Mutation Assay
Test Article: Carbon fibers
ILS No.: 91-40

To the best of my knowledge, this study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 160).

Reviewed by:

Raymond R. Tice, Ph.D.
Division Director, Toxicology

4/14/92
Date

Paul W. Andrews, M.S.
Study Director

4/14/92
Date

CERTIFICATION OF CONTRACT COMPLIANCE

ILS Project No.: A052
Study Director: Paul W. Andrews, M.S.
Study Title: Salmonella Typhimurium/Microsome Reverse Mutation Assay

The contractor, Integrated Laboratory Systems, hereby certifies that, to the best of its knowledge and belief, the technical data delivered herewith under Contract No. DAAA15-91-D-0024 is complete, accurate, and complies with all requirements of the contract.

Raymond R. Tice
Raymond R. Tice, Ph.D.
Division Director

4/14/92
Date

Salmonella Typhimurium/Microsome Reverse Mutation Assay

TABLE OF CONTENTS

Salmonella Typhimurium/Microsome Reverse Mutation Assay

PREFACE

Mutagenicity data evaluations with carbon fibers employing the Salmonella typhimurium plate incorporation assay (Ames test) are presented in this report. The first part of the report contains a study description, criteria for test data acceptance, methods employed in data evaluation, and a summary table of test results. The second part of the report contains an Appendix which provides individual test results with each sample. Item I identifies the sponsor, Item II provides test article information, Item III contains bioassay information, Item IV describes the study management, Item V provides a study description, Item VI describes study results, and Item VII contains the interpretation of the study results. The study results for the test article are given in Tables 1 and 2.

Original experimental data and data analysis diskettes will be archived at Integrated Laboratory Systems, 801-8 Capitola Drive, Alston Technical Park, Durham, NC 27713 (919/544-4589) for a period of five years from the date of report submission to the EPA. Copies of this material will be made available to the sponsor upon request.

The study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 160).

1.0 INTRODUCTION

The Ames/Salmonella mutagenesis test system (Ames et al., 1975) is widely regarded as a valuable prescreen in the identification of mutagenic and carcinogenic chemicals. For several classes of compounds, a highly significant correlation exists between responses in the Ames test and in the rodent bioassay for carcinogenesis (McCann et al., 1975; Sugimura et al., 1976; Purchase et al., 1978; Rinkus and Legator, 1979; Bartsch et al., 1980; Haworth et al., 1983; and Tennant et al., 1987).

2.0 OBJECTIVE

The objective of the study was to investigate the mutagenic potential in the Salmonella/Ames test system of carbon fibers obtained from the U.S. Army Chemical Research, Development, and Engineering Command (USA/CRDEC). The sample was tested on triplicate plates with five tester strains, both with and without metabolic activation.

3.0 MATERIALS AND METHODS

3.1 Test Article Sample

The following sample was received from the USA/CRDEC. The sample was stored at room temperature in the ILS Chemical Repository. Chemical safety and handling data were provided. As per ILS policy, the sample was regarded as hazardous.

ILS #	Sponsor Sample I.D.	Physical Description
91-40	Carbon fibers	black fibers

The entire Carbon fibers sample was extracted with methylene chloride in a standard Soxhlet procedure. The extract was then dried and resuspended in 5.0 ml dimethylsulfoxide (DMSO) for testing in the assay (performed by Webb Technical Group, Inc., Raleigh, NC).

3.2 Bacterial Strains

Five bacterial strains (obtained through the courtesy of Dr. B.N. Ames) were used in these studies. Strains TA1535 and TA100 were used to detect missense mutation (base pair substitutions) and strains TA1537, TA1538, and TA98 for frameshift mutations. All strains were tested for the presence or absence of strain-specific phenotypic markers.

3.3 Metabolic Activation

Metabolic activation was incorporated into the assays by the addition of a post mitochondrial supernatant (S9 fraction) prepared from rat liver homogenates. The S9 fraction (Lot Nos. 0327 and 0358), from male Sprague Dawley rats induced with Aroclor 1254, was purchased from Molecular Toxicology, Rockville, MD.

3.4 Control Chemicals

The control chemicals used in this study were obtained from commercial sources (purity not provided) as follows:

Chemical	CAS Number	Lot Number	Source
dimethylsulfoxide	67-68-5	902873;902300	Fisher
sodium azide	26628-22-8	26F0434	Sigma
2-nitrofluorene	607-57-8	JP03222JJ	Aldrich
9-aminoacridine	90-45-9	75F0276	Sigma
2-aminoanthracene	613-13-8	33F0816	Sigma

3.5 Experimental Protocol

The pour-plate incorporation technique was used with freshly grown bacterial cultures. Simply, the test sample was mixed with 2 ml of molten top agar with 100 ul aliquot of the bacterial culture. Trace amounts of histidine (0.05 mM) and biotin (0.5 mM) were added to the top agar to allow a few divisions of the auxotrophic bacterial strains. To incorporate metabolic activation, 0.5 ml of S9 mix (S9 fraction with an NADP generating co-factor mixture) was added where appropriate. The contents of the test tube were properly swirled on a vortex mixer and poured over previously prepared Vogel-Bonner minimal medium plates. All plates were incubated at 37°C for 48-72 hours.

3.6 Dose Range Selection

A toxicity test was conducted on carbon fibers at 10, 1.0, and 0.1 ul/plate using strain TA100. An aliquot of the test sample was mixed with 100 ul of the tester strain in 2 ml of molten top agar (with and without metabolic activation). The mixture was swirled and poured over previously prepared Vogel-Bonner minimal media. Toxicity was determined by examining the plates for the thinning of bacterial lawn, microcolony formation, or complete growth inhibition.

Carbon fibers exhibited no toxicity in TA100 at the top dose both with and without S9 activation. Hence, the top dose for the assay was set at 10.0 ul/plate.

3.7 Selection of Positive Controls

Positive controls were selected by their specificity to revert tester strains. The missense tester strains (TA1535 and TA100) were tested with sodium azide while the frameshift strains (TA1538 and TA98) were tested with 2-nitrofluorene. The intercalating agent, 9-amino-acridine, was used to test strain TA1537. 2-amino-anthracene was used with all tester strains to evaluate S9 activation.

3.8 Data Acceptance Criteria

The following criteria should be satisfied for accepting test data:

1) The sovent control data should be within acceptable ranges, as follows:

TA1535, TA1537, and TA1538: 4-40 revertants/plate

TA98: 20-65 revertants/plate
TA100: 60-250 revertants/plate

2) The positive controls should exhibit a 3-fold (TA1535, TA1537, TA1538) or 2-fold (TA98, TA100) increase over solvent control data.

3.9 Data Analysis

Revertant colonies were counted on an Artek autocount colony counter. Before counting, the plates were verified for confluent growth of the bacterial lawn. Plates lacking background bacterial lawn or with microcolonies were recorded as such on data sheets but were not used in data analysis. Data were analyzed using the "Salmonella Assay Software" Version 2.3. A test article was regarded as mutagenic if the mean induced revertant number equaled 3.0 or more the mean solvent control number of colonies for strains TA1535, TA1537, and TA1538, and 2.0 or more for strains TA98 and TA100. This increase must be accompanied by a dose-dependent response to increasing test article concentrations. A sample was considered weakly positive if there was no dose response but one or more doses exhibited a doubling/tripling over solvent controls or if there was a dose response but no doses exhibited an appropriately high number of revertants.

4.0 RESULTS AND DISCUSSION

The methylene chloride extract of carbon fibers (ILS # 91-40) exhibited a negative mutagenic response in all five of the Salmonella tester strains both in the presence and absence of exogenous metabolic activation. Detailed results with the test article are supplied in Tables 1 and 2.

5.0 REFERENCES

(1) Ames, B.N. et al. (1975), Mutation Research, 31: 347-364.

(2) McCann, J. et al. (1975), Proc. Nat. Acad. Sci. USA, 72: 5135-5139.

(3) Sugimura, T. et al. (1976), in "Origins of Human Cancer" (Hiatt, Watson, and Winsten, eds.) pp. 1561-1577.

(4) Purchase, I.F.H. et al. (1978), British Journal of Cancer, 37: 873-959.

(5) Rinkus, S.J. and M. S. Legator (1979), Cancer Research, 39: 3289-3318.

(6) Bartsch;, H. et al, (1980), Mutation Research, 76: 1-50.

(7) Haworth, S. et al. (1983), Environmental Mutagenesis, 1: 3-142.

(8) Tennant, R.W. et al. (1987), Science, 236: 933-941.

TABLE 1. Salmonella Mutagenesis Assay of Carbon Fibers (ILS No. 91-40)

MEAN HIS+ REVERTANTS/PLATE

DOSE (ul)	S9	TA1535 mean	TA1535 std	TA1537 mean	TA1537 std	TA1538 mean	TA1538 std	TA98 mean	TA98 std	TA100 mean	TA100 std
0.00	-	29.0	+/- 2.65	14.0	+/- 2.65	16.0	+/- 1.00	30.7	+/- 2.52	75.0	+/- 2.65
0.05	-	28.3	+/- 8.96	10.7	+/- 0.58	23.5	+/- 0.71	26.7	+/- 6.11	98.0	+/- 2.00
0.10	-	31.3	+/- 7.77	13.0	+/- 2.65	22.7	+/- 4.04	26.3	+/- 1.15	103.0	+/- 3.61
0.50	-	26.7	+/- 2.52	15.0	+/- 1.00	19.0	+/- 1.73	27.7	+/- 3.79	103.0	+/- 21.17
1.00	-	31.0	+/- 5.29	9.7	+/- 1.53	14.3	+/- 6.66	26.7	+/- 1.53	110.7	+/- 2.52
10.00	-	32.3	+/- 3.21	11.0	+/- 2.00	17.7	+/- 3.51	28.7	+/- 4.16	89.0	+/- 13.23
POS	-	86.7	+/- 5.03	56.7	+/- 7.77	54.3	+/- 9.07	98.0	+/- 13.00	158.3	+/- 20.03
0.00	+	26.0	+/- 3.46	12.7	+/- 2.08	11.0	+/- 1.00	29.0	+/- 2.65	122.3	+/- 10.07
0.05	+	31.0	+/- 7.55	13.3	+/- 3.21	25.3	+/- 4.16	25.3	+/- 3.06	104.3	+/- 5.86
0.10	+	22.3	+/- 4.51	14.0	+/- 5.29	26.0	+/- 6.08	25.3	+/- 1.53	105.3	+/- 6.43
0.50	+	21.7	+/- 2.52	10.7	+/- 2.31	26.3	+/- 4.16	29.7	+/- 4.04	112.3	+/- 10.12
1.00	+	21.7	+/- 2.08	13.3	+/- 6.81	22.7	+/- 5.03	28.0	+/- 2.00	109.0	+/- 3.61
10.00	+	23.3	+/- 4.51	11.3	+/- 2.08	19.0	+/- 5.00	28.0	+/- 2.00	107.0	+/- 10.82
POS	+	83.0	+/- 16.09	149.3	+/- 41.97	107.3	+/- 8.14	115.0	+/- 8.72	326.0	+/- 20.07

Data presented as the mean of triplicate plates +/- the standard deviation
POS = Positive Control

TABLE 2. Salmonella Mutagenesis Assay of Carbon Fibers (ILS No. 91-40)

DOSE		his+ revertants/plate														
(ul)	S9	TA1535			TA1537			TA1538			TA98			TA100		
0.00	-	32	27	28	11	15	16	17	15	16	28	31	33	78	75	74
0.05	-	33	34	18	11	11	10	24	23	C	28	32	20	96	98	100
0.10	-	25	29	40	10	14	15	25	18	25	27	27	25	107	100	102
0.50	-	24	27	29	14	15	16	21	18	18	32	25	26	79	119	111
1.00	-	33	25	35	11	8	10	10	11	22	27	28	25	108	111	113
10.00	-	30	36	31	13	11	9	16	18	21	24	30	32	99	74	94
POS	-	82	86	92	59	48	63	46	53	64	85	98	111	143	151	181
0.00	+	24	30	24	11	12	15	10	11	12	27	28	32	133	121	113
0.05	+	30	39	24	17	12	11	22	30	24	26	28	22	100	111	102
0.10	+	18	22	27	12	20	10	29	30	19	24	25	27	110	98	108
0.50	+	19	24	22	8	12	12	25	23	31	26	34	29	106	107	124
1.00	+	24	20	21	21	11	8	22	18	28	28	26	30	108	106	113
10.00	+	23	28	19	12	9	13	24	19	14	30	26	28	104	98	119
POS	+	65	88	96	164	182	102	98	111	113	121	105	119	328	305	345

C = contaminated
T = toxic
M = microcolonies
POS = positive control

APPENDIX

In Vitro Mutagenicity Evaluation of Carbon fibers in the Salmonella/Ames Microsome Reverse Mutation Assay

ILS Project No.: ILS A052

Report Date: April 14, 1992

I. SPONSOR: U.S. Army Chemical Research, Development and Engineering Command
Aberdeen Proving Ground, MD 21005

II. TEST ARTICLE INFORMATION

A. Identification: Carbon fibers (ILS No. 91-40)

B. Physical Description: black fibers

C. Stability, Purity, and Chemical Analysis: Not Provided

D. Date Received: November 22, 1991

E. Handling Information: Material handling and safety information was available; treated as a hazardous substance.

III. BIOASSAY INFORMATION

A. Type of Assay: Salmonella/Ames Mutagenicity Assay

B. Study Initiation Date: December 16, 1991

C. Experimental Initiation Date: February 18, 1992

D. Experimental Termination Date: March 13, 1992

E. Study Termination Date: April 14, 1992

F. Archives: Experiment Data Sheets
Media Preparation Log
Data Analysis Diskette
(All records and specimens will be archived at Integrated Laboratory Systems, 801-8 Capitola Drive, Durham, NC 27713)

IV. STUDY MANAGEMENT INFORMATION

A. Study Director: Paul W. Andrews, M.S.

B. Division Director: Raymond R. Tice, Ph.D.

C. Quality Assurance Officer: Kaye Cummings, B.S.

D. Research Assistant: Mary Kimani, B.S.

V. STUDY DESCRIPTION

The test article was evaluated for mutagenic activity in the Salmonella typhimurium plate incorporation assay with five tester strains. The sample was tested directly and in the presence of liver homogenates (S9 fraction) from rats treated with Aroclor 1254. Concurrent positive and solvent controls were run along with five dose points of the test sample. Sterile dimethyl- sulfoxide (DMSO) was used as the diluent in preparing stock solutions. All tests were run in triplicate plates. The doses tested were 10.0, 1.0, 0.5, 0.1, and 0.05 ul per plate.

VI. STUDY RESULTS

The experimental results obtained with the methylene chloride extract of carbon fibers in the Salmonella/Ames Mutagenicity Assay are presented in Table 1 according to strain as the number of his+ revertants per plate. The experiment involving TA1537 without S9 activation needed to be repeated due to an insufficient response from the positive control.

VII. INTERPRETATION OF RESULTS

The results of the assay on the methylene chloride extract of carbon fibers indicate a negative mutagenic response in all five strains both with and without exogenous metabolic activation.

PROTOCOL AMENDMENT

STUDY: Salmonella Typhimurium/Microsome Reverse Mutation Assay

DATE: April 14, 1992

TEST ARTICLE: Carbon fibers

CONTRACT NO.: DAAA15-91-D-0024

ILS PROJECT NO.: A052

AMENDMENT:

Page 1 - Contract No.

The protocol mistakenly states the sponsor contract number as DAAD05-91-D-0024 and should instead be DAAA15-91-D-0024.

Page 1 - MANAGMENT OF STUDY

The protocol states the Research Assistant as Rita Shendrikar. Ms. Shendrikar has since been assigned to different duties and was replaced by Mary Kimani.

Submitted by:

P. Andrews — 4/14/92

Paul W. Andrews, M.S., Study Director — Date

STUDY TITLE
Rodent Bone Marrow Micronucleus Assay

Project No.
ILS A052

Contract No.
DAAA15-91D-0024

Test Article
Carbon Fibers

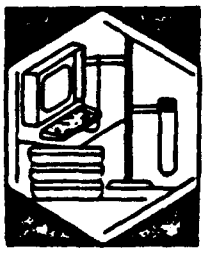
ILS Repository No.
91-40

Final Report Date
February 27, 1992

Sponsor
Mr. Fred K. Lee
U.S. Army, Chemical Research,
Development, and Engineering Command
Aberdeen Proving Ground, MD 21005

Testing Facility
Integrated Laboratory Systems
801-8 Capitola Dr.
Durham, NC 27713

SUBMITTED BY



INTEGRATED LABORATORY SYSTEMS

PO Box 13501, Research Triangle Park, North Carolina 27709.

TABLE OF CONTENTS

STUDY TITLE

Rodent Bone Marrow Micronucleus Assay

PREFACE

Cytogenetic testing with carbon fibers (ILS No. 91-40), employing the Rodent Bone Marrow Micronucleus Assay, is presented in this report. The first part of the report contains a study description, criteria for test data acceptance, methods employed in data evaluation, and a summary of test results. The second part of the report contains the Appendix which provides specific test results. Items I through V of the Appendix identify the sponsor, test article information, bioassay information, project management information, study results, and interpretation of the results. The summarized study results are given in Table 1.

Original experimental data and statistical analysis printouts are archived at Integrated Laboratory Systems (ILS), 801-8 Capitola Drive, Durham, NC 27713 (919/544-4589) for a period of five years from the date of report submission. Stained and coded slides will be held in storage as long as the quality of the preparations affords evaluation. Copies of these records will be made available to the sponsor upon request.

The study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 160).

1.0 INTRODUCTION

During the last decade, the analysis of micronuclei (MN) has gained increased popularity as an alternative to classical chromosomal aberration analysis for detecting clastogenic agents (1-3). The scoring of MN is less subjective and less time consuming than the analysis of chromosomal aberrations, permitting a greater number of cells to be examined for the same extent of effort. This in turn results in greater statistical power. Furthermore, since MN are formed from both acentric chromosome fragments and from lagging, intact chromosomes (1,3), this technique is the most reliable method currently available for evaluating the potential of a chemical to induce either clastogenic and/or aneugenic damage. Among the various in vitro and in vivo MN assays used to detect genotoxic chemicals, the rodent bone marrow micronucleus assay is the most commonly used (1,3). The assay is easily conducted in mice or rats by evaluating the frequency of MN in immature erythrocytes [polychromatic erythrocytes (PCE)] scored in bone marrow (mice or rats) or peripheral blood (mouse only) preparations (1,4-6). The product of recent cell divisions, these enucleated cells are abundant, easily recognizable, and have a

lifetime of approximately two days before maturing into normochromatic erythrocytes (NCE). In the rodent bone marrow assay, the test chemical can be administered by any exposure route used in standard toxicology studies and the exposure regimen can range from single, acute exposure to chronic exposures.

As with other cytogenetic techniques for evaluating genotoxic damage in bone marrow, the timing for sample collection in relation to treatment is a critical parameter. For any agent, the optimal bone marrow sampling time for micronucleated PCE (MN-PCE) induction depends on the mechanism of action, pharmacokinetic considerations, and the extent of erythropoietic suppression. Due to agent-specific differences in peak micronucleus induction, multiple sampling times between 24 and 72 hours are generally recommended after a single treatment to avoid a false-negative conclusion (1,3). A successful method for eliminating the necessity for multiple sampling times is to increase the number of treatments (7). Based on the kinetics of erythropoiesis and the metabolic/distribution properties of most chemicals, a single treatment on three consecutive days offers the greatest opportunity for eliminating the need for multiple sample times. A single sample time protocol minimizes animal usage, decreases the time needed for data collection, and simplifies the statistical analysis.

2.0 OBJECTIVE

The objective of this study was to test the ability of a methylene chloride extract of carbon fibers and/or its metabolites to induce micronuclei in bone marrow cells of male B6C3F1 mice.

3.0 MATERIALS AND METHODS

3.1 Test Article

Carbon fibers was received from the U.S. Army CRDEC and was stored at room temperature in the ILS Chemical Repository. Chemical safety and handling information was not provided and therefore the test article was treated as a hazardous chemical. Based on information supplied by the sponsor, the vehicle selected was corn oil.

ILS Repository No. - 91-40
Sponsor Sample I.D. - Carbon fibers
Physical Description - Black fibers

The entire carbon fibers sample was extracted with methylene chloride in a standard Soxhlet procedure. The extract was then dried and resuspended in 5.0 ml dimethylsulfoxide (DMSO) for testing in the assay (performed by Webb Technical Group, Inc., Raleigh, NC).

3.2 Chemicals and Reagents

The chemicals and reagents used in this study were obtained from the following commercial sources:

Chemical	Source	Lot No.
acridine orange	Sigma	67F-3686
corn oil	Sigma	80H0835
dimethylbenz[a]anthracene	Kodak	G16A
fetal bovine serum	Irvine	300000563
methanol	Fisher	906279
sodium phosphate monobasic	Sigma	37F-0382
sodium phosphate dibasic	Sigma	127F-0130

3.3 Range-Finding Experiment Protocol

The purpose of the range-finding (RF) experiment is to determine the maximum dose for the study. The maximum evaluated dose is that dose which:

a) is the highest dose that induces less than 40% mortality and does not lead to obvious physical stress, or

b) when administered, the dose results in a significant depression of polychromatic erythrocytes (PCE) (not to exceed a 70% depression of the average control value) in the bone marrow, or

c) is the maximum dose which can be administered as a homogeneous suspension without exceeding 2000 mg/kg.

The maximum dose is determined by the following procedure: Using 5 animals/group, mice are treated by intraperitoneal (IP) injection on 3 consecutive days with 0.5x, 1x, and 2x the known, appropriate LD50. If LD50 or other mortality data are unavailable, the initial doses tested are generally 200, 1000, and 2000 mg/kg. Mortality is checked twice daily until animal sacrifice. Forty-eight hours after administering the final dose, surviving mice are sacrificed by CO_2 asphyxiation, and bone marrow smears are prepared. The percentage of PCE in bone marrow is determined. Based on the results obtained, an additional RF study may be conducted using either a higher or lower dose range. Once the maximum dose is established, two additional doses at 0.5x and 0.25x the maximum dose are selected for testing.

3.4 Experimental Protocol

Three IP treatments (dose volume = 0.4 ml) at 24-hour intervals are followed by a single sampling time 24 hours after the final treatment. Mice are killed by CO_2 asphyxiation, assigned random numbers, and bone marrow smears (2 per animal) are prepared.

3.5 Positive Control

A positive control is selected for its ability to induce micronuclei in polychromatic erythrocytes. The positive control for water insoluble compounds is dimethylbenz[a]anthracene (DMBA) at 25.0 mg/kg. DMBA suspended in corn oil was used as the positive control in this study.

3.6 Bone Marrow Slide Preparation

Immediately following CO_2 asphyxiation, the femur is removed. A 22G x 1" needle with a 1 cc syringe is used to push a few drops of fetal bovine serum through the bone marrow cavity, flushing the bone marrow onto a prelabelled slide. A second prelabelled slide is inverted and placed flush to the first. The two slides are rubbed together with a circular motion until the bone marrow is evenly dispersed. The slides are pulled apart and air-dried. After fixing in methanol for 5 minutes and air-drying, slides are stained with acridine orange.

3.7 Micronuclei Scoring

Coded slides (2 per mouse) are scored in numerical order by two scorers. To assess if the test article induced a significant depression in bone marrow erythropoiesis (signified by a reduction in the proportion of PCEs within the total erythrocyte population), the number of PCEs among a total of 200 erythrocytes (100 erythrocytes/animal/scorer) is determined in a continuous field at 1000X magnification. For micronuclei evaluation, 2000 PCEs (1000 PCEs/animal/scorer) are evaluated in continuous field at 1000X magnification for the presence of micronuclei.

3.8 Data Analysis

Data analyses are conducted using a micronucleus assay data management and statistical analysis software (8) developed by ILS for the EPA. In this program, a one-tailed trend test uses pooled data and incorporates a variance inflation factor to account for excess interanimal variability. The trend test is used to determine if a treatment-related increase in MN-PCE frequency

occurred at an alpha level of 0.05 (8,9). An analysis of variance (ANOVA) test based on pooled data is used to determine if a treatment-related difference in the percentage of PCE occurred at an alpha level of 0.05. For statistically significant results, pairwise comparisons between dose groups and the control group are conducted using the appropriate one-tailed (MN) or two-tailed (%PCE) Pearson Chi-Square test (pooled data) to determine the minimal effective dose. A Pearson Chi-Square test is used to evaluate the significance of the positive control MN-PCE and %PCE data.

3.9 Data Acceptance Criteria

A minimum of three surviving mice per dose group is required for test data acceptance. The mean number of MN-PCE in the control group must not exceed 4 per 1000 PCE. The positive control must induce a significant increase in the number of MN-PCEs relative to the negative control.

4.0 RESULTS AND DISCUSSION

4.1 Range-Finding (RF) Experiment

Due to the limited amount the test extract, the range-finding experiment was not conducted.

4.2 Micronucleus Experiment

The doses tested were 2000, 1000, 500, and 250 mg/kg (see Table 1 for group summary data). One animal died at the high dose (2000 mg/kg). Over this dose range, treatment with the carbon fibers extract did not induce a significant increase in MN-PCE ($P = 0.383$). The percentage of PCE was significantly different due to treatment ($P = 0.008$), but not significantly depressed. The positive control, DMBA at 25 mg/kg, induced a significant increase in MN frequency ($P < 0.001$) but did not significantly depress the percentage of PCE ($P = 0.929$).

5.0 CONCLUSIONS

Multiple treatments with a methylene chloride extract of carbon fibers did not result in a significantly increased frequency of MN-PCE and did not significantly depress the percentage of PCE in the bone marrow of male B6C3F1 mice.

6.0 REFERENCES

(1) Heddle, J.A., Hite, M., Kirkhart, B., Mavrournin, K., MacGregor, J.T., Newell, G.W. and Salamone, M.F. (1983) The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutat. Res. 123: 61-118.

(2) Tice, R.R. and Ivett, J.L. (1985) Cytogenetic analysis of bone marrow damage. In: R.D. Irons (ed.) Toxicology of the Blood and Bone Marrow, Raven Press, NY, pp. 119-140.

(3) MacGregor, J.T., Heddle, J.A., Hite, M., Margolin, B.H., Ramel, C., Salamone, M.F., Tice, R.R. and Wild, D. (1987) Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. Mutat. Res. 189: 103-112.

(4) Schmid, W. (1976) The micronucleus test. In: A. Hollaender, (ed), Chemical Mutagens: Principles and Methods for Their Detection, Vol. 4, Plenum, NY, pp. 31-43.

(5) MacGregor, J.T., Wehr, C.M. and Gould, D.H. (1980) Clastogen-induced micronuclei in peripheral blood erythrocytes: the basis of an improved micronucleus test. Environ. Mutagen. 2: 509-514.

(6) Schlegel, R. and MacGregor, J.T. (1982) The persistence of micronuclei in peripheral blood erythrocytes: Detection of chronic chromosome breakage in mice. Mutat. Res. 104: 367-369.

(7) Tice, R.R., G.L. Erexson, C.J. Hilliard, J.L. Huston, R.M. Boehm, D. Gulati, and M.D. Shelby (1990) Effect of treatment protocol and sample time on the frequencies of micronucleated polychromatic erythrocytes in mouse bone marrow and peripheral blood. Mutagenesis 5: 313-321.

(8) Micronucleus Assay Data Management and Analysis System, Version 1.4 (1990) U.S. Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Las Vegas, NV.

(9) Margolin, B.H. and Risko, K.J. (1988) The statistical analysis of in vivo genotoxicity data. Case studies of the rat hepatocyte UDS and mouse bone marrow micronucleus assays, in "Evaluation of Short Term Tests for Carcinogens", Oxford University Press, Oxford, UK, pp 29-43.

TABLE 1: GROUP MICRONUCLEI AND %PCE DATA FOR MICE TREATED WITH CARBON FIBERS (ILS # 91-40)

DOSE (mg/kg)	MN-PCE/1000 PCE[a] MEAN	SEM	%PCE[b] MEAN	SEM	N
DMBA-25	9.90	0.40*	40.9	1.33	5
0	1.40	0.40	37.7	1.85	5
250	SEE NOTE		SEE NOTE		5
500	2.90	0.37	46.0	2.32	5
1000	1.70	0.75	44.7	2.45	5
2000	2.13	1.01	37.0	2.68	4
TREND P-VALUE+	0.383		0.008		

[a] Group mean frequency of MN-PCE per 1000 PCE and standard error of the mean among mice. Data based on 2000 PCE scored per mouse.

[b] Group mean percent PCE and standard error of the mean among mice. Data based on 1000 erythrocytes scored per mouse.

+ One-tailed trend or ANOVA test P-value for MN and %PCE data, respectively. Data analysis based on pooled cells.

* P-value significant at alpha = 0.05.

NOTE: Due to a low number of deaths at the highest dose, it was not necessary to score the 250 mg/kg dose.

APPENDIX

Rodent Bone Marrow Micronucleus Assay

Test Article: Carbon Fibers

ILS Project No.: ILS A052

Report Date: February 27, 1992

I. SPONSOR: U.S. Army, Chemical Research, Development, and Engineering Command
Aberdeen Proving Ground, MD 21005

II. TEST ARTICLE INFORMATION

A. Identification: Carbon fibers (ILS No. 91-40)

B. Physical Description: Black fibers

C. Strength, Stability, Purity and Chemical Analysis: Not provided

D. Date Received: November 22, 1991

E. Handling Information: Treated as a hazardous substance, used safety goggles, impervious gloves, and respirator with an organic filter.

III. BIOASSAY INFORMATION

A. Identification: Rodent Bone Marrow Micronucleus Assay

B. Study Initiation Date: August 19, 1991

C. Experiment Initiation Date: December 20, 1991

D. Study Completion Date: February 27, 1992

E. Archives: Microscope slides and experiment data sheets
Micronucleus scoring data sheets
Data analysis printouts and diskettes
(All records and specimens will be archived at Integrated Laboratory Systems, 801-8 Capitola Drive, Durham, NC 27713)

IV. PROJECT MANAGEMENT INFORMATION

A. Study Director: Paul W. Andrews, M.S.

B. Research Assistant: Rita Shendrikar, M.S.

C. Division Director: Raymond R. Tice, Ph.D.

D. Quality Assurance Officer: Kaye Cummings, B.S.

V. STUDY RESULTS

Multiple treatments with a methylene chloride extract of carbon fibers did not result in a significantly increased frequency of MN-PCE and did not significantly depress the percentage of PCE in the bone marrow of male B6C3F1 mice.

QUALITY ASSURANCE INSPECTION STATEMENT

ILS Project No.: ILS A052
Test Article ID: Carbon fibers
Study Title: Rodent Bone Marrow Micronucleus Assay

Inspection/Audit	Date Performed	Date Reported to Study Director/Management
Data audit	02/18/92	02/19/92 / 02/25/92
Report audit	02/25/92	02/25/92

Kaye Cummings
Kaye Cummings, B.S.
Quality Assurance Officer

2/26/92
Date

CERTIFICATION OF GOOD LABORATORY PRACTICE

ILS Project No.: ILS A052
Test Article ID: Carbon fibers
ILS Repository No.: 91-40
Study Title: Rodent Bone Marrow Micronucleus Assay

To the best of my knowledge, this study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 160).

Reviewed by:

Raymond Tice — Feb 27, 1992

Raymond R. Tice, Ph.D.
Division Director — Date

Paul Andrews — 2/27/92

Paul W. Andrews, M.S.
Study Director — Date

CERTIFICATION OF CONTRACT COMPLIANCE

ILS Project No.: ILS A052
Test Article ID: Carbon fibers
Study Title: Rodent Bone Marrow Micronucleus Assay

The contractor, Integrated Laboratory Systems, hereby certifies that, to the best of its knowledge and belief, the technical data delivered herewith under Contract No. DAAA15-91D-0024 is complete, accurate, and complies with all requirements of the contract.

Raymond Tice
Raymond R. Tice, Ph.D.
Division Director

Feb 27, 1992
Date

PROTOCOL DEVIATIONS

STUDY: Rodent Bone Marrow Micronucleus Assay

DATE: February 27, 1992

TEST ARTICLE ID: Carbon fibers

CONTRACT NO.: DAAA15-91D-0024

ILS PROJECT NO.: A052

DEVIATIONS:

Page 6 - RANGE-FINDING EXPERIMENT

The protocol states that a range-finding experiment will be conducted. However, due to a limited amount of test extract this phase was not performed and an expanded MN experiment was conducted.

Page 7 - RANGE-FINDING EXPERIMENT

Revised Third Paragraph:

Using 5 animals/group, test animals will be treated by IP injection on 3 consecutive days with 200, 1000, and 2000 mg/kg.

Reason for the Deviation:

The original protocol mistakenly called for treatment by gavage.

Page 8 - SCORING

Revised Third Paragraph:

For micronuclei evaluation, 2000 PCEs* are evaluated in continuous field at 1000x magnification for the presence of micronuclei.

Reason for the Deviation:

The original protocol mistakenly indicated that the scoring would be conducted at 100X magnification.

Page 10 - GOOD LABORATORY PRACTICES

Revised Paragraph:

The study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (Federal Register Vol. 54, No. 158, August 17, 1989).

Reason for the Deviation:

The original protocol mistakenly indicated that the study would be conducted in accordance with good laboratory practice regulations for nonclinical laboratory studies.

CRITICAL PHASE AUDIT

No critical phase audit was performed.

Submitted by:

Paul W. Andrews, M.S., Study Director — Date 2/27/92

STUDY TITLE
Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

Project No.
ILS A052

Contract No.
DAAA15-91D-0024

Test Article
Carbon Fibers

ILS Repository No.
91-40

Final Report Date
February 21, 1992

Sponsor
Mr. Fred K. Lee
U.S. Army Chemical Research, Development, and Engineering Command
Toxicology Division
Aberdeen Proving Ground, MD 21005

Testing Facility
Integrated Laboratory Systems
801-8 Capitola Dr.
Durham, NC 27713

SUBMITTED BY

INTEGRATED LABORATORY SYSTEMS

PO Box 13501, Research Triangle Park, North Carolina 27709.

TABLE OF CONTENTS

STUDY TITLE

Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

PREFACE

Clastogenicity data evaluation of carbon fibers (ILS #91-40), employing the In Vitro Chromosomal Aberrations Assay in Chinese hamster ovary (CHO) cells, is presented in this report. The first part of the report contains a study description, criteria for test data acceptance, methods employed in data evaluation, and a summary of test results. The second part of the report contains an Appendix which provides specific test results. Items I through V identify the sponsor, test article information, bioassay information, project management information, study results, and interpretation of the results. The study results summaries are given in Tables 1 through 3.

Original experimental data and data analysis diskettes are archived at Integrated Laboratory Systems (ILS), 801-8 Capitola Drive, Durham, NC 27713 (919/544-4589), for a period of five years from the date of report submission. Stained and coded slides will be held in storage as long as the quality of the preparations affords evaluation. This material will be made available to the sponsor upon request.

The study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 160).

1.0 INTRODUCTION

Structural chromosomal aberrations (CA) are broadly defined as alterations in chromosome morphology. Most CA are deleterious and result in cell death. However, some types (e.g., reciprocal translocations, small deletions, inversions) can lead to altered gene function(s) without an accompanying loss in cell viability. Increasingly, the pivotal role chromosomal mutations play in tumorigenesis is supported by the accumulation of cytogenetic and molecular data on hematological and solid tumors in humans (1-5). While some of the reported chromosomal changes involve aneuploidy of specific chromosomes (6), the majority of chromosomal alterations associated with malignancy have been translocations or deletions (2,5). The emerging premise is that there are specific chromosomal abnormalities responsible for the initiation of malignant transformation in certain cell types. Also, secondary chromosomal changes occur in many malignancies and are associated with a more aggressive progression and with resistance to therapy (7,8). Among the various in vitro systems for detecting clastogenic activity, the CHO Chromosomal Aberrations Assay is

widely regarded as one of the more valuable approaches. The system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals (9).

2.0 OBJECTIVE

The objective of this study was to test the clastogenic potential of carbon fibers (ILS #91-40) and/or its metabolites as measured by their ability to induce structural chromosomal aberrations in CHO cells.

3.0 MATERIALS AND METHODS

3.1 Test Article

The following sample was received from the U.S. Army/CRDEC and stored at room temperature in the ILS Chemical Repository. Based on chemical safety and handling information supplied by the sponsor, the sample was regarded as hazardous. At the request of the sponsor, the final solvent selected was dimethylsulfoxide (DMSO).

ILS Repository No. - 91-40
Sponsor Sample I.D. - carbon fibers
Physical Description - black fibers

The entire carbon fibers sample was extracted with methylene chloride in a standard Soxhlet procedure. The extract was then dried and resuspended in 5.0 ml dimethysulfoxide (DMSO) for testing in the assay (performed by Webb Technical Group, Inc., Raleigh, NC).

3.2 Cell Line

The CHO-K1 cell line is a proline auxotroph with a modal chromosome number of 20, a population doubling time of 10-12 hours, and cloning efficiency of approximately 90%. Stock cultures are maintained at 37°C and 5% CO_2 in Ham's F-12 medium with 10% fetal bovine serum. Cell lines are stored frozen with 10% DMSO in liquid nitrogen. CHO cells were obtained originally from American Type Culture Collection, Rockville, MD.

3.3 Metabolic Activation

Metabolic activation was incorporated into the assay by the addition of S9, the post mitochondrial supernatant fraction of liver homogenates, obtained from male Sprague Dawley rats induced with Aroclor 1254. The S9, supplied by Molecular Toxicology, Gaithersburg, MD, (Lot No. 0327) was stored frozen at -70°C.

3.4 Chemicals and Reagents

The chemicals and reagents used in this study were obtained from the following commercial sources:

Chemical	Source	Lot No.
acetic acid	Fisher	913320
bromodeoxyuridine	Sigma	56F-0767
Colcemid	Irvine	931100209
cyclophosphamide	Sigma	67F-0155
dimethylsulfoxide	Fisher	902873
fetal bovine serum	Irvine	30000563
Giemsa	Gurr	7784800H
Ham's F-12	Irvine	905810516
Hanks Balanced Salt Solution	Irvine	922810214
isocitrate	Sigma	18F-3780
methanol	Fisher	906279
Mitomycin C	Sigma	71H-2505
NADP	Sigma	48F-71206
potassium chloride	Sigma	74F-0018

3.5 Dose Range Selection

Selection of the dose levels was based on toxicity as indicated by the loss of growth potential of the cells. Cells seeded the previous day were exposed to the test article for 22 hours at 37°C in the absence of S9 and for 4 hours in the presence of S9. Four hours after initiation of treatment, bromodeoxyuridine (BrdUrd) was added to all cultures. Following a 26-hour BrdUrd incubation period, with Colcemid present for the last 2 hours, the cells were harvested by scraping with a teflon scraper. The cells were exposed to a hypotonic solution of 0.075 M KCl at 37°C and then fixed using absolute methanol followed by 3:1 methanol: glacial acetic acid. Slides were prepared and processed for sister chromatid differentiation (stained with Hoechst 33258, exposed to blacklight, and then counterstained with 4% Giemsa). Duplicate cultures were evaluated for the percentage of first, second, and third plus subsequent division metaphases, based on 100 metaphase cells per culture, and for the proportion of cells at metaphase (mitotic index), based on 1000 cells per culture.

3.6 Positive Controls

Positive controls were selected for their ability to induce clastogenic damage in CHO cells. Mitomycin C (MMC), at 0.05 and 0.2 µg/ml, was used as the positive control in the nonactivated portion while cyclophosphamide (CP), at 5 and 20 µg/ml, was used as the positive control in the S9 activated portion.

3.7 Experimental Protocol

In the nonactivated study, duplicate cultures were exposed to the test article for 18 hours. At the end of the exposure period, the cultures were washed and media containing Colcemid was added for an additional two hours of incubation. In the S9 activated study, duplicate cultures were exposed to the test article for four hours, washed and incubated for another 16 hours, with Colcemid present for the last two hours. The 20 hour culture time is based on studies by Bean et al. (10) which demonstrate optimal expression of clastogenic activity in the CHO assay at this sample time for a variety of chemicals. Metaphase cells were harvested by scraping with a teflon scraper and collected by centrifugation. After treatment with 0.075 M KCl, the cells were fixed in 100% methanol followed by 3:1 methanol:glacial acetic acid. Cells were dropped onto slides and stained with Giemsa.

3.8 Metaphase Scoring

Coded slides were scored at x1000 magnification without knowledge of the test chemical or dose. In general, 200 metaphase cells from each dose level (100 per duplicate culture) were examined. However, in situations where at least 10 metaphase cells among the first 25 scored were observed to contain at least one CA (excluding gaps), additional metaphase cells were not scored. Metaphase cell selection was based on chromosome morphology, uniform staining quality, lack of overlapping chromosomes, and chromosome number (20 ± 2 centromeres). Individual types of aberrations (i.e., chromatid vs chromosome; gap vs break vs rearrangement), and cells with greater than 10 aberrations (excluding gaps) were recorded separately.

3.9 Data Analysis

For the analysis of cellular proliferation kinetics in the dose determination study, the frequency of first-, second-, and third-generation metaphase cells in each culture was transformed into an average generation time (AGT) where: AGT = BrdUrd exposure duration/Replicative Index (11). The Replicative Index (RI) is equal to 1 times the frequency of first generation metaphase cells + 2 times the frequency of second generation metaphase cells + 3 times the frequency of third generation metaphase cells. The mitotic index (MI) was calculated as the percentage of metaphase cells among 1000 cells.

A one-tailed trend test based on individual cell data was used to determine if a treatment-related increase occurred for the frequency of metaphase cells with at least one CA (excluding gaps) at an alpha level of 0.05 (12). For the test chemical to be declared positive, the trend test must be statistically significant with one or more dose groups expressing at least a doubling in the percentage of damaged cells above the control mean. In addition,

for data resulting in a statistically significant trend test, pairwise comparisons between each dose group and the corresponding control group were conducted using a one-tailed Pearson Chi-Square test to determine the minimal effective dose. A one-tailed Pearson Chi-Square test was used also to evaluate the significance of the positive control data.

3.10 Data Acceptance Criteria

The following criteria were used to determine test data acceptance:

a) The mean number of cells with structural chromosomal aberrations (excluding gaps) in the solvent control cultures must not exceed 6.0%.

b) The number of cells with structural chromosomal aberrations in the positive control should be statistically increased relative to the solvent controls.

4.0 RESULTS AND DISCUSSION

4.1 Dose Range Selection

Over the dose range tested (0.01, 0.05, 0.1, 0.5, and 1.0 µl/ml), CHO cells exposed to the carbon fibers extract in the absence of metabolic activation exhibited neither a significant decrease in the MI nor a significant increase in the AGT (Table 1). Over the same dose range, CHO cells exposed to the carbon fibers extract in the presence of metabolic activation also exhibited no growth toxicity (Table 1). The osmolality of the culture medium at the doses tested in either the absence or presence of metabolic activation was below 400 mOsm (Table 1). A check of medium pH indicated that the pH was not altered beyond the acceptable range (i.e., <6.0 or >8.0). Based on these data, the maximum dose of carbon fibers to be tested in the S9 activated and nonactivated cultures was selected to be 1.0 µl/ml.

4.2 Clastogenic Activity in Nonactivated Cultures

The carbon fibers extract was evaluated for clastogenic activity in the absence of S9 activation at 0.1, 0.25, 0.5, 0.75, and 1.0 µl/ml (Table 2). CHO cells exposed to the two lower doses, 0.1 and 0.25 µl/ml, were not scored for CA. Over the dose range tested, the test article did not induce a significant increase in the percentage of metaphase cells containing at least one aberration ($P = 0.561$). The positive control, MMC, was clastogenic at both 0.05 and 0.2 µg/ml ($P < 0.001$).

4.3 Clastogenic Activity in S9 Activated Cultures

The carbon fibers extract was evaluated for clastogenic activity in the presence of S9 activation at 0.1, 0.25, 0.5, 0.75, and 1.0 µl/ml (Table 3). CHO cells exposed to the two lower doses, 0.1 and 0.25 µl/ml, were not scored for CA. Over the dose range tested, the test article induced a significant increase in the percentage of metaphase cells containing at least one aberration ($P = 0.033$). The lowest effective dose was 0.5 µl/ml. All classes of chromosomal damage, including chromatid- and chromosome-type deletions, and chromatid-type rearrangements, were observed. The positive control, CP, was clastogenic at 5 µg/ml ($P < 0.001$), while excessive toxicity precluding cytogenetic analysis occurred at 20 µg/ml.

5.0 CONCLUSION

The methylene chloride extract of Carbon Fibers was clastogenic in CHO cells in the presence but not the absence of metabolic activation. Under these conditions, the lowest effective dose was 0.5 µl/ml.

6.0 REFERENCES

1. Sasaki, M. (1982) Role of chromosomal mutation in the development of cancer. Cytogenet. Cell Genet. 33: 160-168.

2. Yunis, J. (1983) The chromosomal basis of human neoplasia. Science 221: 227-236.

3. Gilbert, F. (1983) Chromosomes, genes and cancer: A classification of chromosome abnormalities in cancer. J. Natl. Cancer Inst. 71: 1107-1114.

4. Harris, H. (1986) The genetic analysis of malignancy. J. Cell Sci. Suppl. 4: 431-444.

5. Nowell, P.C., and Croce, C.M. (1987) Cytogenetics of neoplasia. In: Development and Recognition of the Transformed Cell. M.I. Greene and T. Hamoaka (eds.) Plenum Press, New York, pp. 1-19.

6. Heim, S., and Mitelman, F. (1989) Primary chromosome abnormalities in human neoplasia. Adv. Cancer Res. 52: 1-43.

7. LeBeau, M.M. and Rowely, J.D. (1986) Chromosomal abnormalities in leukemia and lymphoma: Clinical and biological significance. Adv. Hum. Genet. 15: 1-54.

8. Nowell, P.C., Besa, E.L., Stelmach, T., and Binan, J.B.

(1986) Chromosome studies in preleukemia states. V. Prognostic significance of single versus multiple abnormalities. Cancer 58: 2571-2575.

9. Preston, R.J., Au, W., Bender, M.A., Brewen, J.G., Carrano, A.V., Heddle, J.A., McFee, A.F., Wolff, S., and Wassom, J.S. (1981). Mammalian in vivo and in vitro cytogenetic assays: A report of the Gene-Tox Program. Mutat. Res. 87: 143-188.

10. Bean, C.L., Armstrong, M.A., and Galloway, S.M. (1991) Effect of sampling time on chromosome aberration yield for seven chemicals in Chinese hamster ovary cells. Environmental and Molecular Mutagenesis 17(Supple. 19): 10.

11. Schneider E.L., and Lewis, S. (1981) Aging and sister chromatid exchange. VIII. Effect of the aging environment on sister chromatid exchange induction and cell cycle kinetics in Ehrlich ascites tumor cells. A brief note. Mech. Aging Develop. 17: 327-330.

12. Margolin, B.H., Resnick, M.A., Rimpo, J.Y., Archer, P., Galloway, S.M., Bloom, A.D., and Zeiger, E. (1986). Statistical analysis for in vitro cytogenetic assays using Chinese Hamster Ovary cells. Environmental Mutagenesis 8: 183-204.

TABLE 1. Dose Determination Studies: Replicative Index, Mitotic Index, and Osmolality for Carbon Fibers (ILS # 91-40)

DOSE (ul/ml)	Excluding S9			Including S9		
	AGT	MI	OSMOL	AGT	MI	OSMOL
0.00	9.30	8.50	294	11.18	2.65	306
0.01	9.36	8.35	302	11.39	4.00	310
0.05	9.50	9.95	306	12.30	3.45	314
0.10	9.18	8.25	312	11.93	5.20	318
0.50	9.28	7.05	319	11.22	4.45	310
1.00	9.21	9.20	315	11.32	4.15	317

Abbreviations: AGT = BrdUrd Incubation time/RI, where the RI = Replicative Index = the frequency of first generation metaphase cells + 2 x the frequency of second generation metaphase cells cells + 3 x the frequency of third generation metaphase cells.
Data based on 100 metaphase cells per culture.
MI = Mitotic Index = Number of metaphase cells per 1000 cells.
OSMOL = osmolality presented in mOsms.

TABLE 2. Frequency of Chromosomal Aberrations in CHO Cells Without Metabolic Activation Treated With Carbon Fibers (ILS# 91-40)

DOSE (ul/ml)		NO. CELLS	ABERRATION TYPES											CA/Cell		%DC		
			G'	G*	B'	B*	DM	TR	QR	Dic	Rg	CR	>10	+gaps	-gaps	+gaps	-gaps	
MMC-0.2	*	100	4	0	29	10	0	3	6	0	2	9	0	0.630	0.590	45.00	43.00	**
MMC-0.05	*	200	10	0	26	8	0	4	3	0	0	6	0	0.285	0.235	21.00	19.00	**
SOLVENT		200	5	0	3	2	0	0	0	1	0	0	0	0.055	0.030	4.50	2.50	
0.10		not scored																
0.25		not scored																
0.50		200	7	0	3	1	0	0	0	1	0	1	0	0.065	0.030	5.50	3.00	
0.75		200	2	0	2	1	0	0	0	0	0	1	0	0.030	0.020	3.00	2.00	
1.00		200	5	0	1	2	1	0	0	0	0	1	0	0.050	0.025	5.00	2.50	
													TREND TEST P VALUE				0.561	

Abbreviations: G',G* = chromatid and chromosome gaps, respectively;
B',B* = chromatid and chromosome breaks, respectively; DM = double minute;
TR = triradial; QR = quadriradial; Dic = dicentric; Rg = ring;
CR = other complex rearrangment; >10 = more than 10 aberrations per cell;
CA/cell = number of aberrations per cell; %DC = percent of metaphase cells with at least one aberration.

*Dose in ug/ml

**Significantly different from control data at alpha = 0.05

TABLE 3. Frequency of Chromosomal Aberrations in CHO Cells With S9 Activation Treated With Carbon Fibers (ILS# 91-40)

DOSE (ul/ml)		NO. CELLS	ABERRATION TYPES G'	G°	B'	B°	DM	TR	QR	Dic	Rg	CR	>10	CA/Cell +gaps	CA/Cell -gaps	%DC +gaps	%DC -gaps	
CP-20	*	not scorable																
CP-5	*	100	6	0	33	12	1	6	10	0	1	11	1	0.900	0.840	47.00	46.00	**
SOLVENT		200	4	0	3	2	0	0	1	0	0	1	0	0.055	0.035	5.00	3.00	
0.10		not scored																
0.25		not scored																
0.50		200	9	0	13	0	0	2	1	0	0	3	0	0.140	0.095	13.50	9.00	**
0.75		200	8	1	14	4	0	2	1	0	0	1	0	0.155	0.110	13.50	10.00	**
1.00		200	7	0	13	1	1	0	0	0	1	0	0	0.115	0.080	9.00	6.50	**
													TREND TEST P VALUE				0.033	

Abbreviations: G',G° = chromatid and chromosome gaps, respectively;
B',B° = chromatid and chromosome breaks, respectively; DM = double minute;
TR = triradial; QR = quadriradial; Dic = dicentric; Rg = ring;
CR = other complex rearrangment; >10 = more than 10 aberrations per cell;
CA/cell = number of aberrations per cell; %DC = percent of metaphase cells with at least one aberration.

*Dose in ug/ml

**Significantly different from control data at alpha = 0.05

APPENDIX

Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

Test Article: Carbon Fibers

ILS Project No.: A052

Report Date: February 21, 1992

I. SPONSOR: U.S. Army Chemical Research, Development, and Engineering Command

II. TEST ARTICLE INFORMATION

A. Identification: Carbon Fibers (ILS #91-40)

B. Physical Description: black fibers

C. Purity, Stability, and Chemical Analysis: not provided

D. Date Received: November 22, 1991

E. Handling Information: Treated as a hazardous substance; used safety goggles, impervious gloves, and respirator with an organic filter.

III. BIOASSAY INFORMATION

A. Identification: Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

B. Study Initiation Date: December 16, 1991

C. Experiment Initiation Date: December 16, 1991

D. Experiment Completion Date: February 5, 1992

E. Study Completion Date: February 21, 1992

F. Archives: Microscope slides and experiment data sheets
Chromosome aberration score sheets
Data analysis printouts and diskettes
(All records and specimens will be archived at Integrated Laboratory Systems, 801-8 Capitola Drive, Durham, NC 27713)

IV. PROJECT MANAGEMENT INFORMATION

A. Study Director: Paul W. Andrews, M.S.

B. Research Associate: Jeanne Leitner, B.S.

C. Division Director: Raymond R. Tice, Ph.D.

D. Quality Assurance Officer: Kaye Cummings, B.S.

V. STUDY RESULTS

The methylene chloride extract of carbon fibers was clastogenic in CHO cells in the presence but not the absence of metabolic activation. Under these conditions, the lowest effective dose was 0.5 µl/ml.

QUALITY ASSURANCE INSPECTION STATEMENT

ILS Project No.: A052
Test Article ID: Carbon fibers (ILS # 91-40)
Study Title: Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

Inspection/Audit	Date Performed	Date Reported to Study Director/Management
Phase inspections:		
Target Cell Preparation- + and -S9, toxicity	12/16/91	2/05/92
Target Cell Preparation- (CA)	1/13/92	2/05/92
Data audit	2/12/92	2/12/92
Report audit	2/12/92	2/12/92

Kaye Cummings
Kaye Cummings, B.S.
Quality Assurance Officer

2/20/92
Date

CERTIFICATION OF CONTRACT COMPLIANCE

ILS Project No.: A052
Test Article ID: Carbon fibers
Study Title: Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

The contractor, Integrated Laboratory Systems, hereby certifies that, to the best of its knowledge and belief, the technical data delivered herewith under Contract No. DAAA15-91-D-0024 is complete, accurate, and complies with all requirements of the contract.

Raymond Tice
Raymond R. Tice, Ph.D.
Division Director

2/21/92
Date

CERTIFICATION OF GOOD LABORATORY PRACTICES

ILS Project No.: A052
Test Article ID: Carbon fibers
ILS Repository No.: 91-40
Study Title: Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

To the best of my knowledge, this study was conducted in accordance with the EPA Good Laboratory Practice regulations, 40 CFR Part 160.

Reviewed by:

Raymond Tice
Raymond R. Tice, Ph.D.
Division Director, Toxicology

2/21/92
Date

Paul Andrews
Paul W. Andrews, M.S.
Study Director

2/21/92
Date

PROTOCOL DEVIATIONS

STUDY: Chromosome Aberrations Assay in Chinese Hamster Ovary (CHO) Cells

DATE: February 21, 1992

TEST ARTICLE ID: Carbon fibers (ILS# 91-40)

CONTRACT NO.: DAAA15-91D-0024

ILS PROJECT NO.: A052

DEVIATION:

Page 1 - Contract Number

The contract number is mistakenly listed as DAAD05-91D-0024 and should be DAAA15-91D-0024.

Page 4 - TREATMENT OF TARGET CELLS

Revised First Paragraph:

In the nonactivation study, CHO cells will be exposed for 18 hours at 37 ± 1°C in 5 ± 1% CO_2 in air. The treatment medium will be removed at the end of the exposure period, the cells washed with Hank's Balanced Salt Solution, and growth medium containing Colcemid (final concentration of 0.1 µg/ml) will be added for an additional two hours of incubation.

Revised Second Paragraph:

In the S9 activated study, CHO cells will be exposed for 4 hours at 37 ± 1°C in 5 ± 1% CO_2 in air. At the end of the exposure period, the treatment medium will be removed, the cells will be washed with Hank's Balanced Salt Solution, refed with growth medium containing 10% serum and returned to the incubator for an additional 16 hours. Colcemid will be added during the last two hours of incubation at a final concentration of 0.1 µg/ml.

Reason for the Deviation:

Recently published data indicate a culture time of 20 hours to be optimal for the expression of clastogenic activity in CHO cells for a variety of chemicals.

Submitted by:

Paul Andrews

Paul W. Andrews, M.S., Study Director

2/21/92

Date